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Volume 502

HYPOXIA

From Genes to the Bedside

Edited by Robert C. Roach,
Peter D. Wagner,
and Peter H. Hackett

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HYPOXIA

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Preface

The International Hypoxia Symposia (IHS) meet every other year to bring together international experts from many fields to focus on and discuss the state of the art in normal and pathophysiological responses to hypoxia. Representatives from six continents and 32 countries joined together in March 2001 for four days of intense scientific discourse in the dramatic mountain setting of Jasper Park Lodge.

In 2001 we also had the privilege of honoring John T. Reeves as a friend, colleague, mentor and inspiration to many in the field. Robert Grover's personal tribute to John "Jack" Reeves is the first chapter in this volume.

As organizers of the IHS, we strive to maintain a near quarter century tradition of presenting a stimulating blend of clinical and basic science talks. Topics covered in 2001 include gene-environment interactions, a theme developed in both a clinical context regarding exercise and hypoxia, and in native populations living at high altitudes. Skeletal muscle angiogenesis is the focus of a stimulating review, and further focus on skeletal muscle in hypoxia is given in two papers debating the benefits and mechanisms of high altitude training. An update on high altitude pulmonary edema clearly is the state-of-the-art review on this complex pathophysiological problem. Erythropoietin is reviewed in a group of three papers ranging from an up to date review, to new insights into the biology of the erythropoietin receptor and to a clinical review of athletes and erythropoietin abuse. An exciting paper on the neuromodulation of high altitude weight loss sheds new light on this decades old problem. The role of stress proteins in physiological responses to hypoxia is carefully presented in a pair of papers, with further links to erythropoietin physiology. Transepithelial sodium transport in the central and peripheral nervous system, in the lung, and in the kidney is the focus of three excellent papers. The latest advances in cardiorespiratory control in hypoxia are explored in three papers from a basic science perspective. The final section explores frontiers in important areas of hypoxia research. The first reviews the world literature on intermittent hypoxia training in humans, the second presents new data on emerging

therapies for pulmonary hypertension, including a fascinating section on gene transfer. And finally, the pathophysiology of chronic mountain sickness is explored in revealing detail. The abstracts from the 2001 meeting were published in *High Altitude Medicine and Biology* 2(1), 2001.

We hope that this collection of papers especially prepared for this volume allows us to share with a broader audience some of the intellectual excitement that embodies the spirit of the Hypoxia meetings.

Robert C. Roach, Peter D. Wagner, Peter H. Hackett, Editors, June 2001.

Acknowledgments

The 12th International Hypoxia Symposium was a rewarding experience due to the outstanding faculty and the lively participation of our largest group of participants. At this, our second meeting as the organizers, we were especially pleased that the experience known as the Hypoxia Meetings can continue to prosper. We remain always thankful for the kind and wise guidance of Charlie Houston and Geoff Coates, the originators of the Hypoxia meetings.

Ms. Sharron Studd from the Office of Continuing Medical Education at McMaster University deserves special thanks from us for doing a great job taking care of the organizational details.

In 2001 we had the generous support of a number of organizations and individuals, including the U.S. Army Research and Development Command, The John Sutton Fund, The International Society for Mountain Medicine, and Drs. Allen Cymerman, Annalisa Cogo, Luciano Bernardi, and Otto Appenzeller. And thanks are also due to numerous others who freely gave of their time and energy to make the meeting such a resounding success.

Please join us by the light of the full moon in February 2003 at the Banff Mountain Centre in Banff, Alberta for the 13th International Hypoxia Symposium.

*Robert Roach and Peter Hackett, Chairmen
International Hypoxia Symposium (www.hypoxia.net)*

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Chapter 1

John T. Reeves, MD

Reflections on an old friend

Robert F. Grover

Professor Emeritus of Medicine, University of Colorado School of Medicine, Denver, CO, USA



John T. Reeves, M.D.

One of the highlights of each Hypoxia Symposium is the honoring of an outstanding individual for his or her career contributions to the field. For this 12th International Symposium, John T. (Jack) Reeves, MD, was selected to receive this honor. No one is more deserving, and I say this having had the privilege of Jack's friendship for nearly half a century.

That friendship began in Denver in the mid 1950's. The University of Colorado School of Medicine and its teaching hospital, Colorado General Hospital, had launched one of the few programs in cardiology that included surgical repair of

congenital heart defects in children employing the technique of whole body hypothermia pioneered by Henry Swan. Many of these patients had pulmonary hypertension. When I was placed in charge of the cardiac diagnostic and research (CVP) laboratory I realized that dealing with this associated problem required a better understanding of the control of the pulmonary circulation. Logically, then, the study of the pulmonary circulation became one of my major interests. In July of 1957, the chief of cardiology, S. Gilbert Blount, Jr., brought one of his new fellows, Jack Reeves, to train with me in the lab. From the outset we hit it off, and soon

Jack was sharing my fascination with the pulmonary circulation. That has remained a major aspect of his scientific career ever since.

Jack and I soon learned that airway hypoxia is one of the most common and most potent factors causing pulmonary hypertension, and of course hypoxia is the *sine qua non* of high altitude. One of our colleagues, Giles F. Filley, told us about a condition in cattle at high altitude known to ranchers as *brisket disease* in which severe pulmonary hypertension leads to heart failure. We learned veterinarians at our neighboring institution, Colorado State University (CSU) in Fort Collins were conducting research on brisket disease, and soon we were collaborating with them. This marked the beginning of Jack's (and my) interest in high altitude physiology, a second sustained aspect of his research career.

At this point it is relevant to point out this collaboration between the investigators in Denver and in Fort Collins has lasted more than 40 years. This remarkable and fruitful association is due largely to Jack's interest not only in physiology but also in the training opportunities it has presented to young research fellows at both institutions. Jack loves to teach, and he is an inspiration to young people interested in both research and its application to clinical medicine.

In 1960 Jack and I designed our own study of cattle taken to 3900 m on Mt. Evans, with Donald Will from CSU as a co-investigator. It was during this intense project that Jack showed me his amazing stamina and skill for organizing daily tasks. We would drive from home at 1600 m in Denver to our base of operations at 3100 m where we would spend the night. Next morning at dawn we would drive up to the experimental site at 3900 m, feed our livestock, often reestablish the precarious water system, fill several wheelbarrows with manure, then fire up the electric generator, and set up our recording equipment. Only then were we ready to begin another series of extensive hemodynamic and respiratory studies on several steers. Being a 'cowboy' was strenuous work, especially at that altitude, and all to be accomplished by noon. Then back down to 3100 m to begin the lab work. After developing rolls of photographic records, Jack would dive in to the laborious task of making manual measurements of pressures and calculating cardiac outputs. Once completed, you would find him hunkered over a microscope doing blood counts. If that weren't enough, he then began drawing graphs of the results with comparisons to earlier data. His energy seemed without limit. Finally, after working non-stop for 12 hours at altitudes above 3000 m, we would pack up for the drive back to Denver. No matter what he did, he immersed himself in the task with cheerful enthusiasm. He has always been like that.

Jack remained in Colorado four years before returning to his native Kentucky where he joined the cardiology faculty in Lexington with Borys Surawicz. But his heart never left Colorado. He could not forget the

challenge of research, the thrill of new discovery, and the satisfaction of sharing these adventures with eager young trainees. He and I stayed in close contact, and he even returned to Colorado in 1964 for another joint research project, this time with human subjects, high school athletes.

Which brings me to another facet of Jack's personality. When considering an experimental procedure on another human, Jack insisted on undergoing the procedure himself first. When we were studying cardiac output during changes in posture and during exercise, he insisted I perform heart catheterization on him first, and with a catheter in his pulmonary artery he climbed the endless treadmill. Then during the study of exercise limitation at high altitude in young athletes, we decided we needed pilot data on arterial saturation. Again, I was called upon to place the rigid Cournand needle in Jack's brachial artery. Only then would Jack seek a volunteer to undergo the same procedure. One young man did agree, and Jack proceeded to probe for what proved to be an elusive artery. Finally, after some inevitable mutual anxiety, he was successful. Jack turned to the young man and said 'Brian, you're a *real brick*!' Brian looked dismayed and we wondered why. It turned out Brian had never heard the word *brick* used as a compliment and he misinterpreted the *b*! We all had a good laugh over that one.

In 1972, with new funding from NIH, I was able to offer Jack a faculty position back in Denver, so after eleven years in Kentucky he accepted the offer eagerly. Once again he was free to pursue his true passions, research and teaching. Over the next few years he launched a series of major research projects dealing with such diverse topics as the reduction in cardiac output following ascent to high altitude (collaborative effort with investigators at USARIEM using their sophisticated chamber facility at Natick MA), the causes of acute mountain sickness (once-in-a-lifetime expedition to Pheriche in Nepal close to Everest Base Camp), and the mechanisms by which chronic hypoxia stimulates structural remodeling of the pulmonary vascular tree (collaborative study with CSU using their excellent hypobaric chamber facility).

There followed the monumental Everest II project involving multiple investigators (again using the USARIEM chamber facility at Natick). This challenged not only Jack's physical and scientific abilities but also all of his diplomatic skills to avert an outright mutiny among the investigators. In the end, the project yielded a group of landmark papers on human adaptation to extreme altitude. In fact Jack has a daunting bibliography including several hundred technical reports, many reviews, numerous chapters in textbooks. He is also the editor of the proceedings of many symposia including the Grover Conferences on the Pulmonary Circulation that he established in collaboration with E. Kenneth Weir when I retired.

Jack Reeves is not only an amazingly productive investigator but also a fine human being who loves his fellow man. Rarely do you meet an

individual who derives more genuine pleasure from his interactions with his associates. No one is a stranger to Jack. He will enter a room full of people and proceed directly to introduce himself to men or women he has never met before. Quickly, a conversation opens and within minutes he knows their names, where they come from, and what they are interested in. He has a talent for remembering names, a talent few of us have including myself. And when he meets that person again days or even months later he will greet him or her by name and recall the personal details he acquired during that first encounter.

Jack loves to browse through poster sessions at scientific meetings. Often these posters are manned by young students eager to present their latest findings to the world. But at the same time the students are often shy and even intimidated by some of the elderly icons circulating among them. Jack will approach a lonely but hopeful student and ask him or her to tell him about their poster. He has told me nothing gives him more pleasure than to watch the smile spread over the young face and see his or her eyes light up as an enthusiastic explanation begins. Someone has shown interest in their work!

Jack is a natural storyteller. To illustrate this talent, consider the following. In 1991 Jack and I were part of a research team studying the role of the beta adrenergic nervous system on human adaptation to high altitude. We were working in the Maher Research Laboratory located at 4300 meters on the summit of Pikes Peak, Colorado. This outstanding facility is operated by the United States Army Research Institute of Environmental Medicine (USARIEM) and here we have collaborated with investigators from Natick many times.

Our subjects were a group of male college students from Stanford University. The research protocol called for each subject to exercise sitting on a bicycle ergometer after first having multiple catheters placed in various veins and arteries. This is no picnic for any subject, and on one particular day the investigators were having more difficulty than usual with a nervous fellow named Henry. After multiple attempts to intubate his femoral artery, we noticed Henry had turned pale, had begun to perspire, and his pulse was slowing. Vagal syncope appeared imminent. Quickly, Jack sized up the situation, stepped in and waved everyone off. Now recall Jack is originally from the small town of Hazard in the backcountry of Kentucky. From that background he began to spin a tale about a good ol' boy named Buford Pusser from the hills of Kentucky. Jack's animated style captured Henry's attention immediately, allowing Jack to lead him through a tangled web of absurd circumstances. Not only was Henry enthralled, but soon everyone else in the laboratory was taken in also. Jack had become the Pied Piper leading all of us, his spellbound throng, through his fanciful maze. Henry's anxiety soon vanished, his color returned, and the day was saved. But in the

process, all activity in the lab had been suspended. Jack had to break his spell so work could resume.

Jack and his wife Carol are true humanitarians. They have spent many weeks each year in Ukraine as part of a medical missionary group called NADIYA struggling to establish the concept of family practice as the source of primary medical care for a people formerly dependent on the bureaucratic Soviet Union. Not only has this been physically demanding on the Reeves, but it has been wrought with frustration, often leaving them discouraged. You have to admire them for taking on such a major challenge. Jack does indeed love his fellow man.

It would not be an exaggeration to say Jack Reeves personifies the concept of the Renaissance man. His interests and knowledge extend far beyond science and medicine. His artistic nature finds expression in the lens of an old reflex camera, and while working in the dim red glow of the darkroom coaxing razor sharp black and white images from the developing pan. In this way he provides counterpoint to his wife's acclaimed watercolors. His writing skills extend far beyond dry technical reports. Most recently he and I prepared and edited a collection of biographies laced with glimpses into the personal lives of pioneers in high altitude research ('Attitudes on Altitude'). Jack is an avid reader of non-scientific literature. To share his literary 'discoveries' he has published a list of his personal favorite short stories under the title 'Good Words in Small Books.' Titles range from *Goodbye Mr. Chips* to *The Book of Job* from the Bible. Jack established criteria for inclusion in his list, the first of which was 'They must be fun for me to read.' *Fun* is the operative word. Jack believes whenever possible life's activities should be enjoyable. Why does he do research? Because it is *fun*. Why does he teach? Because it is *fun* as well as rewarding. And when asked what was different about retirement, he replied 'I no longer get paid for having fun in the laboratory.'

Editor's Note: We are pleased to include an outstanding scientific contribution to this volume by Dr. Reeves. See chapter 27, starting on page 419.

Chapter 2

Genetic lessons from high altitude

Charles S. Houston

Professor Emeritus of Environmental Medicine, University of Vermont, Burlington, VT, USA

Abstract: What lessons can we learn from mountain sickness or wellness that may apply to our patients? Does hypoxia affect us similarly regardless of its cause? Millions of individuals are as hypoxic from congenital or acquired heart or lung disease as healthy individuals may be at altitude. Certain adjustments enable many of such patients to lead nearly normal lives. I will compare their adjustments to those seen in the acclimatization of healthy persons to altitude. Some living organisms use strategies which enable them to tolerate a degree of hypoxia lethal to man. Are there lessons we can learn from them? In my short talk I will raise a few provocative questions, and these will lead me to discuss the relevance of basic research to the resolution of vexing human ailments. When does commercialization of research become a form of intellectual prostitution?

Key words: genes, lung disease, commercialization

*A first mist and a planet
A crystal and a cell
A jellyfish and a saurian
And caves where cavemen dwell
Then a sense of awe and beauty
And a face turned from the sod
Some call it Evolution
But others call it God.*

INTRODUCTION

In 1906, Thomas Hunt Morgan concluded from his work on fruit flies that certain little particles he saw in each cell were the basis of life. He was building on the earlier work of Mendel, Correns, Tschermak and De Vries. A few years later Wilhelm Johanssen coined the name gene for the tiny units in chromosomes that Morgan had predicted would determine the form an organism would take.

Six years after Morgan's work, Jacques Loeb published his magisterial work effectively destroying the theory of a vital force by showing that sterile sea urchin eggs could be artificially fertilized by chemicals alone: the unfertilized eggs contained all the genes they needed to mature. Though attacked by some he was widely acclaimed for demonstrating a purely mechanistic origin of life. Loeb would have been delighted a century later when the unfertilized ovum from a ewe was enucleated and then stimulated by an electric spark to grow into the world famous Dolly – with all her mother's genes. Now a young bull has been cloned from his father's sperm alone. These wonders continue but no one has yet found the spirit or soul which we associate with life.

About the time Jacques Loeb was writing, Thomas Ravenhill, doctor for a mine high in the Andes, published the first description of altitude illnesses. His paper was lost for half a century but World War II stimulated research in hypoxia which has grown to a flood. In 1925 Sir Joseph Barcroft, a pioneer in altitude physiology, suggested that observations of mountain sickness might be relevant to patients hypoxic from illness at sea level. Though he did not mention genes, from what we are learning of their role, I believe he was right.

The process of growth, familiar to high school students, is similar in most animals and I will refer only to humans. The process begins within hours of fertilization, when the female ovum forms a cell that quickly divides into two identical and potentially viable cells. Each can grow into an individual with unique characteristics, inherited from genes from both male and female, or as we have seen, from either alone under special conditions. The sperm has many more genes than does the female ovum. In humans, usually only one of the two infant cells survives. Its genes dictate the growth of the individual.

Recent evidence suggests that sickness or wellness at altitude may be influenced by genes as well as by environment or circumstances. Some individuals may have a genetic heritage that enables them to tolerate hypoxia, to acclimatize to a point; others will be sick or even die at altitude. But many genes are pleiotropic and may affect other individual traits as well, just as traits may be affected by more than one or a few genes.

We know that the speed and severity of exposure to altitude can be decisive and we know that most individuals can acclimatize within some limits of time and altitude. We know that some individuals resident for generations at altitude, differ genetically from other ethnic groups long resident at similar altitudes – but these are the highest altitudes to which any human can adapt permanently. We can identify these genes and will some day be able to transplant them – perhaps to benefit hypoxic patients at sea level. Such ethnic gene research is still in its infancy and is certain to shed light on other hypoxic conditions.

Barcroft did not recognize the same mountain sicknesses we know today, but he would be excited to learn that a gene found in persons specially susceptible to HAPE has been identified. It appears to be responsible for failure to generate NO-Synthase, and the hypothesis is that individuals who cannot generate sufficient NO are vulnerable to HAPE. That same gene has been found in some patients with primary pulmonary hypertension, raising interesting possibilities for treatment.

Mammals must match oxygen supply to demand, so it may be highly significant that a Hypoxia-inducing factor 1 (HIF-1) influences erythropoietin expression and affects oxygen sensing by heme. Since this alters oxygen uptake and release it could be important in many hypoxic conditions.

Among dozens of studies of angiotensin converting enzymes, two have shown that veteran high altitude mountaineers, as well as some Olympic champions have an “endurance” gene (ACE 1 variant) that seems to give them an edge over their peers. This has been challenged by others. Now that we are able to insert specific genes into an ovum, does this suggest that someday we may breed an Everest summitter or an undoped Olympic star? This seems unlikely because of the many other factors involved in super-efforts. Yet such genes might benefit hypoxic patients. Only yesterday this was science fiction but it is less so today.

A rare blood abnormality has been linked to a specific gene that causes over-production of red blood cells, one of the changes that enhance acclimatization – but which is also a major factor in chronic mountain sickness. Might it also be a culprit in polycythemia vera?

Especially promising is the work being done with stem cells to shape growth, replace defective genes or add new ones, raising unprecedented possibilities because our birthright stem cells are poly-directional – during their first seven to ten days of life each can become any type of cell. Placental and umbilical cord blood is rich in stem cells which can be transplanted to or grow into any tissue.

I believe that these few examples are only a tiny and incomplete sample of genetic physiology which may be of great benefit both at altitude and for hypoxic problems at sea level. Barcroft was right and ahead of his time.

Let me mention two over-simplified examples from patients with chronic obstructive pulmonary disease: the Pink Puffer copes with his oxygen deficit by over-breathing, thereby decreasing the dilution of incoming air with residual air in the lungs. In this he resembles the Tibetan high altitude resident. The Blue Bloater, on the other hand, tries to adjust by increasing his oxygen carrying capacity to sustain tissue oxygenation. He resembles the Andean altitude native. Can we consider these to be genetic lessons from high altitude? Here are wonderful fields for research.

What we now know about genes has increased so swiftly in the last decade that we are overwhelmed by the ethical, political, medical, and financial issues raised. We know more than we understand; knowledge has outstripped wisdom. And we are only at the beginning. Forty inheritable diseases have been identified, and each has been linked to a specific gene. The popular press describes new developments in banner headlines. The prospects for prevention and treatment are vast. Dozens of genome research centers employ thousands of scientists in a booming industry. What will we do in this brave new world? The arena is booby-trapped with politics and money and research scientists are leading contestants.

I suggest to you that the relevance of research is not irrelevant. It is no longer enough that the search for truth is an end in itself. We must ask, why our work matters, and who is to benefit, and in this fast moving world, when. It took twenty-five years of research before iodine was added to salt to prevent goitre and cretinism. The needless delay caused millions of avoidable disabilities. Several decades of research proved the dangers of smoking, but political and economic pressure prevented the actions which would have saved thousands of lives. Denial, prejudice and economics have delayed application of years of research in HIV/AIDS while the disease has spread wildly throughout the world.

But today we seem to be going too far. Applied research is beginning to dominate and to distort "pure" research. In a world which shows signs and symptoms of crashing, using our knowledge becomes critical to survival – but for public, not for private good. Unfortunately an ugly virus – Greed – is prevalent throughout the world. Medical research has become a lucrative industry. Distinguished scientists campaign covertly for a Nobel prize, often pre-empting the work of others. Academic success can lead to rich contracts. with pharmaceutical companies.

In 1980 the courts ruled that genes can be patented, starting a gold rush that has made genetic research a bonanza for individuals, research centers and multinational corporations. Two groups have identified the 30,000 genes that make up the human genome, and published the code in the scientific press. One of the teams was funded by taxpayers, and the data made available to all on the World Wide Web. The second team was supported by venture capital and has applied for many patents which already yield riches.

Blood from an individual with a rare disease, cells from a patient's cancer, tissues from neurological defects – all have been patented. A test for breast cancer has been patented and cannot be used for any patient without paying royalties - not to the patient whose cells produced the test but to the company holding the patent.

True, research is frightfully expensive and sponsors have every right to recoup investments and make a modest profit, and certainly for every success there are many failures. Yes, scientists have every right to be appropriately compensated in a world where youths become billionaires in high tech ventures or multi-millionaires in competitive sports. And no, we don't want to stifle genius or control entrepreneurs. But is it ethical for a scientist or a company to patent human genes? To sell for profit human tissue taken from patients?

Genetic engineering opens a Pandora's box of riches and corruption. The scientific community must quickly find solutions for these moral issues before the triumphs of some become disasters for many.

Chapter 3

Genes, environment, and exercise

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Abstract: The definition of the term “environment” has broadened in the past 40 years to include knowledge generated from sequencing genes. Studies on animal responses to the environment have expanded to include selective lifestyle behaviors. Environmental lifestyle components interact with susceptibility genes to pass a threshold of biological significance such that a disease requires clinical treatment. Examples of environmental-gene interactions producing cystic fibrosis and asthma are described. The contributing role of physical inactivity to the epidemic of type 2 diabetes is presented with some of its underlying effectors. A lack of contractile activity by skeletal muscle is associated with less GLUT4 protein in the sarcolemma and a lower glucose uptake into the muscle. The pathways by which contractile activity signals an increase in glucose uptake differs from insulin signaling, but is remarkably similar to how hypoxia stimulates muscle to increase its glucose uptake.

Key words: skeletal muscle, physical inactivity, glucose uptake, hypoxia

*“...the physiology of an organism cannot be described
without considering its environmental interactions.”*

—C. Ladd Prosser (23)

INTRODUCTION

According to its classical definition, “environment” is comprised of biotic (other organisms) and abiotic (non-living physical and chemical components) factors that affect an organism. However, the meaning of the term “environmental” has broadened in the last 40 years. In 1966, Edgar

Folk's textbook, *Introduction to Environmental Physiology* (9) defined environmental physiology as the "study of healthy mammals in relation to their physical environment..." Importantly however, Folk argued that an organism's behavior also warrants consideration in analyses concerned with environmental physiology. According to him, the role of behavior in thermoregulation is plainly illustrated when the "cold mammal curls itself into a ball, and the 'hot mammal' stretches out to increase surface area (9)." The chapters in Folk's book reflect this definition in describing the interactions of various species with their physical and biological microenvironments. Although this conference is focused on cold temperature and low barometric pressure, inclusion of behavior in the broader definition of environmental physiology necessitates the application of additional tools and concepts in the study of organisms subjected to these physiological surroundings.

One concept that has received recent attention is that of environmental gene interaction. Since Folk's definition of environmental physiology in 1966, our knowledge of gene expression and regulation has significantly increased. The search terms "environment gene interaction" on Medline resulted in 1288 citations, approximately 140 of who were published in the year 2000. Many, but not all, of these citations discussed clinical disorders such as chronic obstructive pulmonary disease, asthma, cancer, osteoarthritis, cystic fibrosis, etc. Thus, the term "environmental physiology" has evolved from Folk's definition to include lifestyle behaviors that alter gene expression, subsequently resulting in altered physiological or pathophysiological phenotypes.

As high altitude research has been the interest of many pulmonary scientists, we have selected lung diseases to illustrate environmental-gene interactions as the disease example. Crystal (6) recently reviewed potential future research in lung disease and his concepts will be described next. The respiratory epithelium is daily exposed to an environmental burden of infectious agents, allergens, and particulates. Nonetheless according to Crystal (6), only a fraction of these individuals develop lung disease, and the extent of disease resulting from the inhalation of these agents also varies. He contends that it is the interaction among the similar environments, variable genetics, and dissimilar host defenses that account for the different disease susceptibilities among individuals. Using cystic fibrosis as an example, Crystal contends that the dysfunction of electrolyte transport by a mutant *CFTR* gene in itself does not have a major direct effect *per se*. Rather, it is the chronic airway infection with *Pseudomonas* and other organisms, and the consequent inflammation of the airway epithelial surface that are responsible for the clinical manifestations of cystic fibrosis (27). Crystal (6) wrote: "In this context, it is unlikely that the genetic abnormalities of the *CFTR* mutations would have significant clinical pulmonary consequences without the environmental challenge of *Pseudomonas* and other organisms."

Presenting other examples, Crystal (6) explains "...the general principle that genetic variations determine individual susceptibility to pulmonary disease, and that the manifestations of lung disease require a complex interaction between a challenge from the environment, and host responses in the lung." Thus in an extension of Folk's definition of environmental physiology (9), all inhaled substances (as with cold temperature) are now classified as environmental modulators of gene function.

A second example of lung diseases, asthma, better illustrates the inclusion of lifestyle behavior in the current definition of environmental gene interaction. There is a genetic predisposition to asthma, which is fundamental to its etiology, but poorly understood because of the tremendous influence of the environment over disease expression (2). Thus, asthma is multifactorial, in that disease expression is influenced by interactions between multiple major and minor genes, and modulated by interacting non-genetic factors (e.g. environment) (2). Environmental factors important in asthma include cigarette smoking (active/passive), allergen exposure, viral and bacterial respiratory illness, occupation, and possibly diet (26). Consequently, many of these environmental components are functions of an individual's lifestyle. Further, just as it is possible to manipulate genes for curing disease, the power of primary disease prevention through alteration of the environment should not be ignored.

Current research emphasis seems to favor genes over environment. This is understandable with the rapid sequencing of genomes, including the human, and the subsequent excitement about molecular medicine. For example in a recent review, Crystal (6) wrote that the research opportunities and forecasts for lung diseases included the identification of: mechanisms that modulate the timing of lung gene expression, modes of gene transfer into the lung, susceptibility genes, lung stem cells, inflammatory receptors in the lung, and virulence sequences in pathogen genomes as well as the development of antiproteases and antioxidants, gene-based vaccines, and aerosol administration of lung therapeutics. Noticeably absent is any mention of exploring the mechanisms of environmental-gene interactions to provide evidence-based medicine for primary disease prevention. We believe that altering the environment to prevent disease before it occurs is as important as exotic methods to cure or stabilize disease once it develops.

Most post-natal human clinical disorders have some environmental basis. For example, only 2% of human disease is produced by single gene mutations (28). On the other hand, the other 98% of human clinical disorders can be attributed to complex, multi-factorial causes involving multiple genes and their environmental interactions (28). The common chronic diseases of adults (coronary heart disease, diabetes mellitus, and essential hypertension) have been described by Beaudet et al. (3) as an interaction of multiple genes with various environmental factors in the etiology of individual cases to produce familial aggregation without a simple Mendelian pattern (3). Their

concept of the underlying genetic network is that there are constitutional (polygenic) components, consisting of multiple genes at independent loci, whose effects interact in a cumulative fashion. Beaudet et al. (3) further enunciate the concept that any given individual will inherit a particular combination of disease susceptibility genes that produces some relative risk that may combine with an environmental component to cross a "threshold" of biologic significance such that the individual is affected with overt clinical disease. The multifactorial genetic disease, maturity-onset diabetes, and its interaction with a single environmental factor, physical inactivity, will be discussed in the remainder of this review as an example of how the interaction between an environmental component and an individual's genetic profile is of sufficient biological significance to result in a clinical disorder.

The incidence of maturity-onset diabetes has been increasing steadily in the US for 70 years. Davidson wrote that the prevalence of diagnosed diabetes increased 10-20-fold in the US from 1930-1980 (7). Davidson (7) felt that an increased prevalence of overweight individuals played a more significant role in the greater incidence of maturity-onset diabetes than did increased longevity and more sensitive diagnostic criteria. The number of adults known to have maturity-onset diabetes increased 9-fold between 1958 and 1998 (5). The prevalence of diabetes in the US increased 41% from 1990 to 1999 with an increase of 6.2% in 1999 alone (19,20). A letter authored from the CDC stated: "Diabetes is clearly a growing public health threat in the U.S. This update is consistent with our earlier prediction of the epidemic nature of diabetes (19)." This increase in prevalence of maturity-onset diabetes cannot entirely be due to gene mutations in millions of individuals since 1930. Rather 100% of the increase in maturity-onset diabetes since 1930 is due to a change in the environment with no change in gene sequence. Existing maturity-onset diabetes susceptibility genes have thus combined with environmental components to cross a "threshold" of biologic significance such that individuals are affected with clinical disorders such as type II diabetes.

Another aspect of the epidemic of maturity-onset diabetes is the increase of this adult disease in children and the elimination of the term "maturity" from the disease. Based upon genes, maturity-onset diabetes is an adult disease, but based upon the environment for children, maturity-onset diabetes is a pediatric disease. Harrison's 1980 textbook (10) states that maturity-onset diabetes usually begins on middle life (> 40 yrs) or beyond. In 1987 Vague (25) wrote that type 2 diabetes mellitus is commonly diagnosed after the age of 45 years and that maturity-onset diabetes was rare in the pediatric population (22). Vague (25) further states that it was for this reason that type 2 diabetes was previously called maturity-onset diabetes. Of 43 cases of maturity-onset diabetes diagnosed before the age of 30, Vague (25) indicated that 42 patients had a relative body weight lower than 120% of normal. The increase in maturity-onset diabetes in adolescents reported to

occur in the 1990's was associated with body weights greater than 120% (21). For example, at the Children's Hospital in Cincinnati, Ohio from 1982-1994, a ten-fold increase in maturity-onset diabetes type 2 diabetes occurred among adolescents (21). Although the reasons for increased maturity-onset diabetes is not entirely clear, Pinhaus-Hamiel and Zeitler (22) speculate that the rise in prevalence of this disorder is likely related to current trends in childhood obesity and changes in eating and exercise behavior.

Sedentary lifestyle increases the incidence of maturity-onset diabetes. Sedentary, female nurses who were 4-65 yrs old had a 67% higher rate of diabetes as compared to a group who walked a minimum of 3 hrs/wk at a speed of 3.0-3.9 miles/hr (14). Finnish men 42-60 years old with a low-risk for maturity-onset diabetes had a 2-fold greater incidence if they partook in moderately intense (≥ 5.5 METs) for < 40 min/wk, or no intense exercise, as compared to those had moderately intense (≥ 5.5 METs) for > 40 min/wk (17). Similar trends were observed in men who had a high-risk for maturity-onset diabetes. The incidence of diabetes was 3-fold greater in these high-risk individuals when they were less active than high-risk group exercising at ≥ 5.5 METs for > 40 min/wk (17). Thus, epidemiological data show that physical inactivity increases the incidence of maturity-onset diabetes.

A physiological basis for these epidemiological data exists as reductions in physical activity have been shown to produce insulin resistance, a forerunner of maturity-onset diabetes. Continuous bed rest for 3 days by healthy young men produced insulin resistance (16). Well-trained subjects who stopped training for 10 days had a 100% higher maximal rise in plasma insulin concentration in response to a 100-g oral glucose load than when the subjects were exercising regularly (13). Despite the increased insulin levels, blood glucose concentrations were higher after 10 days without exercise (13). A single bout of exercise by untrained individuals increases the sensitivity and responsiveness of insulin-stimulated glucose uptake that lasts 2 days, but is gone by 5 days (18). Skeletal muscle contributes to these whole body increases in insulin resistance associated with decreased physical activity. For example, the normal increase in 2-deoxyglucose uptakes into the mouse soleus muscle with increasing doses of insulin was lost when hindlimbs were immobilized for only 1 day (24). Thus in response to a decrease in physical activity (an environmental factor), increases in insulin resistance occurred. Increases in insulin resistance if occurring for long enough periods of time permit a crossing the "threshold" of biologic significance such that the individual is diagnosed with maturity-onset diabetes.

The biological bases for these observations are beginning to be understood. GLUT4 is the predominant mammalian facilitative glucose transporter isoform expressed in insulin-sensitive tissues including skeletal muscle. When normal skeletal muscle is both inactive and in the presence of fasting blood insulin levels, glucose uptake by the muscle is low compared

to either exercise or insulin stimulation. However, acute aerobic contractile activity and insulin signal the translocation of GLUT4 transporters to the sarcolemma and T-tubules in normal skeletal muscle by distinct signaling pathways (11). Whereas insulin signals GLUT4 translocation via IRS, PI3-kinase, and Akt signaling, aerobic contractions do not use this pathway, but signal GLUT4 translocation via AMP kinase. Whereas insulin-stimulated GLUT4 translocation in skeletal muscle of individuals with type 2 diabetes is defective, a single bout of exercise results in the translocation of GLUT4 to the plasma membrane in skeletal muscle of individuals with this disorder (15). Thus an environmental factor such as physical activity alters a physiological response by a different mechanism than an endogenous hormone. A potential speculation is that some exercise-signaling pathways may signal changes in gene expression by distinctly different mechanisms to hormonal signaling.

Before 1990 it was believed that aerobic contractions and hypoxia used the same signaling pathway to stimulate increases in glucose uptake by skeletal muscle. In 1991, Cartee *et al.* (4) showed that hypoxia, like contractions stimulated muscle membrane glucose transport by increasing sarcolemmal GLUT4 content, presumably by the translocation of GLUT4 from an intracellular pool to the cell surface. In addition, they demonstrated that the maximal effects of hypoxia and insulin on glucose transport activity were additive, whereas the effects of exercise and hypoxia were not, providing evidence suggesting that hypoxia and exercise stimulate glucose transport by the same mechanism (4). Hypoxia has also been shown to use a signaling pathway that bypasses the insulin receptor, IRS-1 and IRS-2, and the activation of PI 3-kinase (1). However, Derave and Hespel (7) propose that at least some steps in the pathway for stimulation of muscle glucose uptake by contractions and by hypoxia must be different due to their report that submaximal hypoxia and contractions act as additive stimuli to muscle glucose uptake. Thus, a second environmental stimuli, hypoxia, independently affects GLUT4 translocation.

SUMMARY

In summary, the environmental components of environmental gene interactions are important in determining whether a crossing of the "threshold" of biologic significance occurs and the individual is diagnosed with maturity-onset diabetes. This is significant because a more balanced research approach between environment and genes needs to be taken. Environmental manipulations of glucose uptake employ distinct signaling pathways from insulin. This is important because it emphasizes that some

environmental manipulation of gene expression may occur by mechanisms unique from those considered as conventional.

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Chapter 4

Skeletal muscle angiogenesis

A possible role for hypoxia

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Abstract: Skeletal muscle is one of the most plastic tissues in the body. Repeated exercise causes several muscle adaptations, among which the development of additional capillaries (angiogenesis) is prominent. Conversely, inactivity and some chronic diseases result in loss of muscle capillaries. Since (endurance) exercise depends on adequate O₂ supply, it is reasonable to hypothesize that hypoxia occurring within muscle during exercise may provide the stimulus to angiogenesis. However, there are other potential stimuli including physical effects of increased muscle blood flow, or of muscle contraction; release of molecules such as NO that could transcriptionally activate angiogenic growth factors; and perhaps changes in the biochemical milieu of the muscle cell such as acidosis. This brief review will address evidence collected to date mostly at the molecular biological level that does in fact implicate reduced intracellular PO₂ as a major stimulus to the angiogenic process resulting from exercise. In particular, it is shown that VEGF message and protein are increased in muscle with exercise, more so in hypoxia, and that HIF-1 α correlates with VEGF as would be expected if hypoxia were the major stimulus. In addition, we show that muscle intracellular PO₂ falls to very low levels during exercise (3-4 Torr), providing a degree of hypoxia compatible with a strong role for low PO₂ in angiogenic growth factor response. However, the definitive experiments using acute gene manipulation to establish a cause and effect relationship between hypoxia and muscle angiogenesis remain to be performed.

Key words: vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF-1 α), nitric oxide (NO), proton magnetic resonance spectroscopy, exercise

INTRODUCTION

The performance of endurance exercise cannot occur without adequate O₂ supply to skeletal muscle mitochondria (36). This in turn requires appropriate O₂ conductance at every step in the O₂ transport chain from the environment to the muscle mitochondria (where O₂ is used in oxidative phosphorylation to generate ATP for muscle contraction). While adequate ventilation, pulmonary gas exchange and cardiac function are well-known to be important factors whose quantitative performance affects the level of O₂ transport (34), the possible controlling role of the muscle microcirculation in affecting mitochondrial O₂ availability remains debated (22,23). While important at sea level, these concepts are even more important at altitude due to environmental hypoxia.

The degree of muscle capillarity could be critical to O₂ delivery for two reasons: First, the rate of outward diffusion of O₂ from the capillary into the myocyte will depend on the amount of capillary surface area available (Fick's law of diffusion). Second, the amount of time available for O₂ unloading from red cells flowing through the muscle capillary bed is given by the ratio of capillary blood volume to muscle blood flow rate. Thus, for a given flow rate, this transit time will vary in direct proportion to the aggregate volume of the capillaries.

Considerable experimental evidence shows that in healthy normal athletic humans (and other species), the rate of diffusive transport of O₂ from muscle microvascular red cells to the mitochondria is indeed limited and contributes significantly to the maximal rate of O₂ supply (and thus exercise intensity) that can be achieved (15,26,27). The early work of Krogh a century ago (18) focused on another aspect of Fick's law of diffusion – diffusion distance from the red cell to the mitochondria – as the critical element of muscle O₂ diffusion limitation. However, recent work (2,13,14,17) suggests that how much capillary network is present (and not diffusion distance) is the important determinant of muscle O₂ transport conductance. Current thought therefore is that maximal endurance exercise is limited not only by O₂ transport through the arterial tree (which depends on adequate pulmonary and cardiovascular function) but also on muscle O₂ transport conductance that in large part determines ability to extract O₂ from the blood (33). In turn, this conductance is determined mostly by muscle capillarity.

Muscle capillarity is plastic. It has been known for many years that endurance training results in increased capillarity (angiogenesis) in the exercised muscles (28) while inactivity and some chronic disease states (such as chronic renal failure (21) and chronic heart failure (31)) result in reduced capillarity. There has been little study of the cause and effect links between activity and angiogenesis in muscle. Presumably, one or more physiological or biochemical consequences of exercise set in motion a chain

of events that lead to capillary proliferation, but the details are only now being investigated.

This paper will summarize the key data that implicate muscle intracellular hypoxia as a likely major stimulus to the angiogenic process. Such a link, while attractive teleologically as a classical negative feedback mechanism for adaptation, is by no means a given. First, one must ask what angiogenic machinery exists within muscle and if it responds to both exercise and hypoxia. It needs to be established whether intracellular hypoxia develops within myocytes during exercise. Competing hypotheses for other angiogenic stimuli need to be explored, and finally, the ultimate test of the hypoxic theory will require deletion of those genes encoding angiogenic proteins that are hypoxia-inducible and examination of the morphological consequences for capillary response to exercise and hypoxia.

DOES ANGIOGENESIS OCCUR IN RESPONSE TO EXERCISE, DO CAPILLARIES DISAPPEAR IN CHRONIC DISEASE?

Endurance exercise training is well-known to result in angiogenesis at the muscle capillary level (28). Figure 1 shows, from the work of Tom Adair (20), remarkable increases in capillary number around fibers in the rabbit anterior tibialis muscle after 60 days of electrical stimulation. Several weeks of endurance exercise training in man has shown similar if not so dramatic results. Conversely, Painter and Moore (21) have shown reduced capillarity in the skeletal muscle of patients with chronic renal failure (Figure 2), a finding also seen in chronic heart failure (31). There is little question concerning the ability of the muscle to respond bidirectionally to presumed augmentation and withdrawal of angiogenic stimuli. There is considerable question about the basic mechanisms underlying these changes.

DOES CAPILLARITY AFFECT O₂ AVAILABILITY TO THE MITOCHONDRIA?

Not detailed in this paper, there is a considerable body of evidence supporting a key role for muscle capillarity in determining muscle O₂ conductance. Thus, both Bebout (2) and Hepple (14) showed that in canine skeletal muscle, O₂ conductance was a function of capillary number rather than diffusion distance.

**Effect of long-term electrical stimulation on
capillary growth in rabbit anterior tibialis muscle**

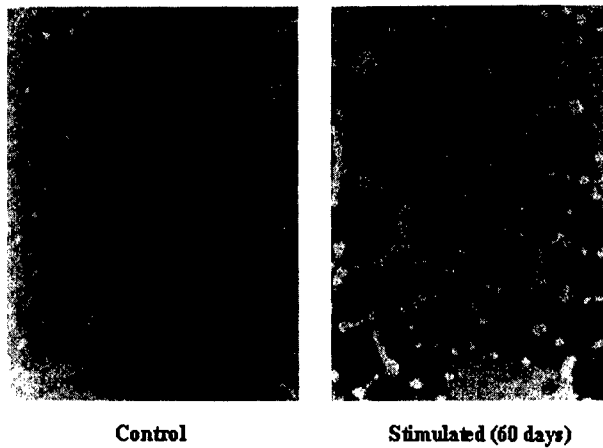


Figure 1. Angiogenesis in rabbit tibialis anterior after 60 days of electrical stimulation. From Adair (20). Capillaries (empty white circles surrounding each muscle fiber) are greatly increased after stimulation.

Theoretical studies of Groebe and Thews (13) show that most of the red cell to mitochondrial PO_2 gradient occurs over the very short distance from the red cell to the myocyte cell wall. Honig and coworkers (17) have shown that muscle myoglobin O_2 saturation, and thus PO_2 , are very low during exercise (PO_2 about 2 Torr when intravascular PO_2 is about 100 Torr at the arterial and about 20 Torr at the venous end of the capillaries), compatible with Groebe's predictions. Using proton magnetic resonance spectroscopy to measure myoglobin O_2 saturation, we have found in man that intracellular PO_2 is also very low (about 3 Torr) during exercise (25), with similar intravascular PO_2 values as above. If the great majority of the PO_2 drop from the muscle microvascular red cell to the mitochondrion happens between the red cell and the sarcolemmal membrane, this provides a strong argument that most of the resistance to O_2 diffusion in muscle is related to the capillary, either through a limited surface area or limited transit time. Either results from insufficient numbers of capillaries.

HOW IS MUSCLE CAPILLARITY REGULATED?

There is a large literature on all aspects of angiogenesis (443 reviews in the last 5 years), but it mostly pertains to tissues other than muscle. This cannot be reviewed here. Most is related to cancer biology, to wound

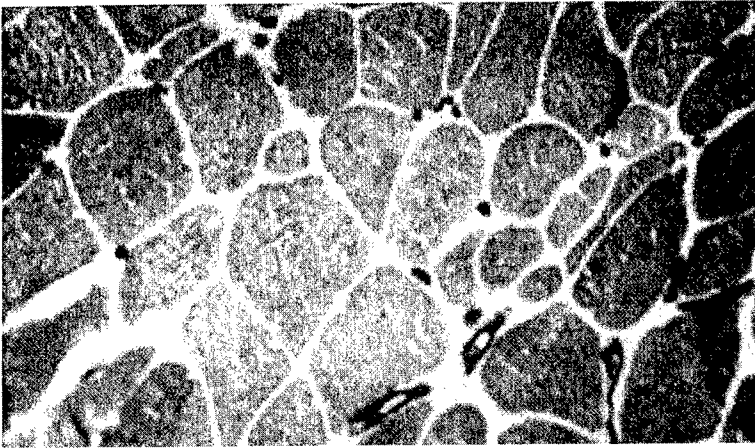


Figure 2. Rectus femoris muscle stained for capillaries by alkaline phosphatase in a patient with chronic renal failure. From Moore et al (21). Vascularity is poor, with some fibers apparently devoid of any visible capillaries.

healing and to embryonic development, but the topic permeates essentially all tissues and organs in health and disease, with common basic threads. Of most importance to the present case, it is thought that local hypoxia is a powerful angiogenic stimulus (30). For example, as rapidly growing tumors outstrip their vascular supply, the tumor cells become hypoxic and this causes them to secrete angiogenic growth factors that initiate a complex angiogenic process whose end result is a greater microvascular supply with alleviation of the hypoxia (11,12).

That is also a natural hypothesis for the angiogenic process in skeletal muscle, and this will be the focus of the remainder of this paper. It is the stimuli to putative angiogenic growth factors in particular that currently take center stage, as the factors that cause increased levels of these proteins that initiate and enable the angiogenic process. The question therefore becomes which growth factors are the most important candidates underlying capillary growth responses to exercise. Any review of angiogenesis will reveal a long list of known and putative angiogenic growth factors. However, the key members are Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (bFGF), with roles for many other molecules such as Transforming Growth Factor β -1 (TGF β ₁) and their various cell-surface receptors.

As far as skeletal muscle is concerned, the key player seems to be VEGF. To keep focus, we will not further discuss other growth factors in this paper, since published work suggests an order of magnitude lower response to exercise for other growth factors in muscle compared to VEGF (4,35). This is consistent with the importance of VEGF in other systems and situations,

cancer biology and embryonic development in particular. This should not however be taken as suggesting that VEGF is the sole mediator of angiogenesis. There will also be no discussion of the basic nature of VEGF itself – this is well described in the literature (5,8).

VEGF, EXERCISE AND SKELETAL MUSCLE

Is there VEGF in mammalian skeletal muscle? Figure 3 shows by *in situ* hybridization the presence of VEGF mRNA accumulated in the sub-sarcolemmal region in rat gastrocnemius muscle (4). It is seen clearly in all fibers.

Does VEGF in skeletal muscle respond to exercise with an increase in its message and protein? Figure 4 shows that indeed there is a 3-4 fold rise in VEGF mRNA in rat gastrocnemius after a one hour run at $20 \text{ m} \cdot \text{min}^{-1}$ (4). Moreover, it is an early response, with peak levels seen immediately after exercise is completed. Thereafter, VEGF mRNA levels return to baseline after about 4 hours. Data not shown (4) document that the degree of response correlates with exercise intensity.

ACTIVATION OF MUSCLE VEGF AND HYPOXIA

Does muscle VEGF mRNA increase with hypoxia? This is perhaps the critical question in the present context. A negative answer would suggest that hypoxia is not the stimulus to VEGF upregulation. However, a positive answer cannot be taken as proof of direct effect of hypoxia on VEGF. Thus, hypoxia may well affect VEGF by indirect mechanisms such as increased muscle blood flow and/or vasodilator release. Figure 5 shows that VEGF mRNA is higher in hypoxic rats breathing 12% O_2 than in rats breathing room air (4). This is evident not only at rest, but also after exercise. Exercise at a given absolute intensity increases VEGF mRNA slightly in normoxia and more so in hypoxia.

Before proceeding with further exploration of VEGF responses to exercise at the molecular level, we should pause and ask if exercise reduces intracellular PO_2 in muscle and thus provides a basis for its being the important stimulus to VEGF. Figures 6 and 7 show that intracellular PO_2 drops to 1-3 Torr during moderate and heavy exercise. Figure 6 comes from the work of Honig et al (17) using myoglobin spectrophotometry to measure myoglobin O_2 saturation in dog muscle, while Figure 7 comes from that of Richardson et al (25) who used proton MRS to measure myoglobin O_2 saturation in man. The results are similar, and do provide the likely environment required for hypoxia to play a role in VEGF activation.

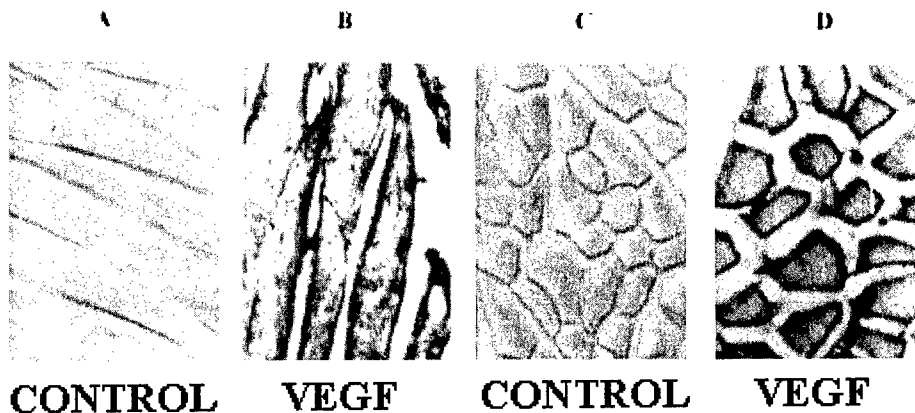


Figure 3. In situ hybridization of resting dog gastrocnemius showing VEGF mRNA in sub-sarcolemmal accumulation.

If VEGF transcription is upregulated by hypoxia, it would be expected from the work of Semenza (9) that Hypoxia-Inducible Factor (HIF-1 α) should be increased as well. This is because HIF-1 α is turned on by hypoxia and is known to bind to the VEGF gene, promoting transcriptional activation of VEGF, and an increase in VEGF mRNA. Figure 8 shows by Western blot that HIF-1 α protein levels in rat gastrocnemius rise 6-fold after an hour of intense, fatiguing electrical stimulation (32). Moreover, there is a corresponding 6-fold rise in VEGF mRNA. That similar stimulation in hypoxia does not further increase either HIF-1 α or VEGF levels may mean that maximal activation of the system has occurred already in normoxia in this particular experiment. Note also that the intracellular PO₂ levels shown in Figure 7 are not that different between normoxia and hypoxia in man, and that we have not done what would be very labor intensive experiments to examine the dose-response relationships over the exercise range in normoxia and hypoxia between HIF-1 α , VEGF and intracellular PO₂. Such would be needed to establish the reasons for similar responses in normoxia and hypoxia shown in Figure 8. The main point of Figure 8 is that HIF-1 α protein levels and VEGF mRNA levels increase similarly during exercise, consistent with a determining role for hypoxia in VEGF regulation.

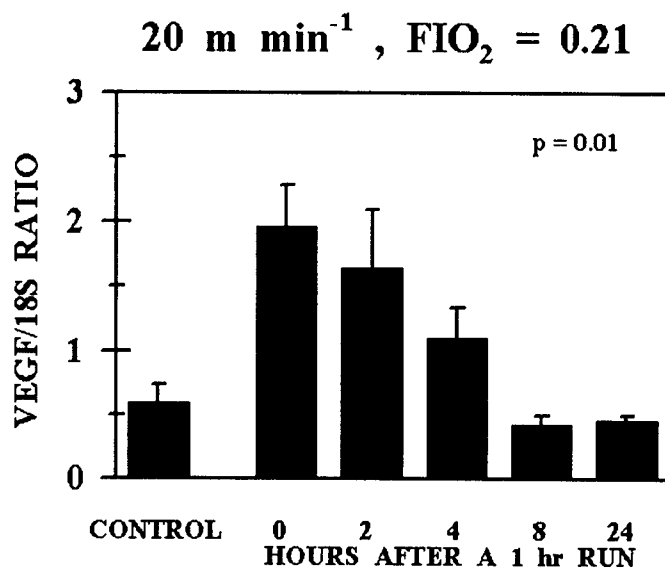


Figure 4. Time course of VEGF mRNA response to a single one hour bout of exercise (rat gastrocnemius muscle). Message abundance is increased 3-4 fold immediately after exercise, and falls back to baseline over about 4 hours.

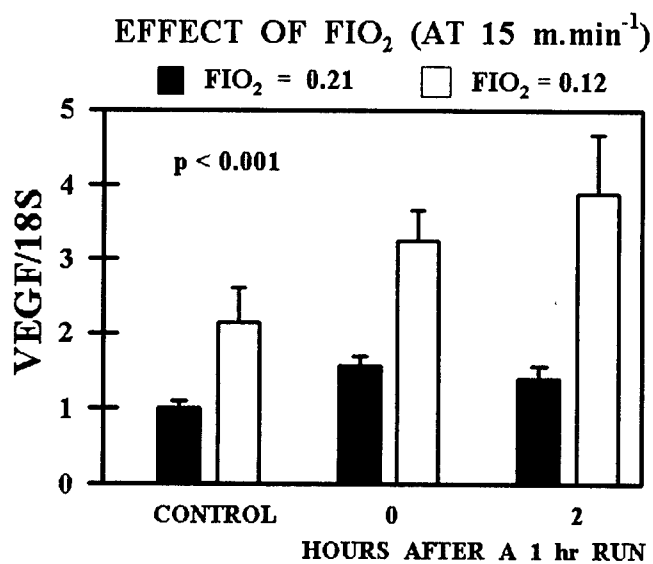


Figure 5. Hypoxia (rat breathing 12% O₂) doubles VEGF mRNA at rest and after one hour of exercise, compared to the same exercise level in normoxia.

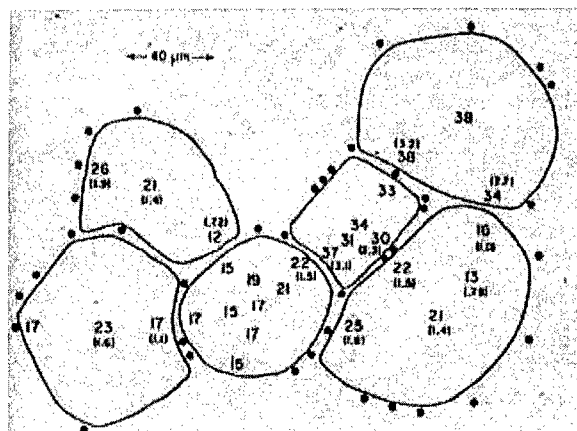


Figure 6. Frozen myoglobin spectroscopic data (17). Six canine fibers are shown in cross-section, with their surrounding capillaries (black dots). Intracellular O₂ saturation and calculated PO₂ in brackets are shown to be very low in all fibers.

Figure 8 also shows that hypoxia alone (rats breathing 12% O₂) increases VEGF mRNA about 2-fold, just as in prior work shown in Figure 5. However, there is no change in HIF-1 α protein to explain this. This result is compatible with the known effect of hypoxia on VEGF mRNA stabilization (6). Thus, we would propose that hypoxia of this degree only prolongs the half-time of already present VEGF mRNA, while exercise results in new transcription of VEGF mRNA. To support this hypothesis, it would be important to show less hypoxia in resting muscle when FIO₂ is 0.12 than during exercise in normoxia. Figure 9 shows that this is the case, at least in normal man. The data reflect myoglobin O₂ saturation measured by proton MRS as a normal subject breathed 12% O₂ (25). Each solid point reflects a 20 second set of data. The Y-axis depicts the MRS signal, which reflects myoglobin desaturation. At rest there is no signal different than at rest during normoxia and there is no desaturation. Intracellular PO₂ must be at least 10-15 Torr, about the upper limit of detection of PO₂ given the signal to noise ratio and the P₅₀ of the myoglobin-O₂ dissociation curve of 3.5 Torr. This is compared to the rapid desaturation to about 50% seen on exercise and which translates as shown in Figure 7 to PO₂ values of 2-3 Torr. Taken together, the MRS and molecular data suggest that moderate hypoxia is sufficient to activate VEGF mRNA stabilization while more severe hypoxia is necessary to activate VEGF transcription.

Increased VEGF message is the first step, but if translation does not follow, VEGF will not be effective in promoting angiogenesis. Figure 10 shows from the same samples as in Figure 8, that there is indeed increased VEGF protein in response to the higher levels of message (32). The relative increases of message and protein are similar at rest in hypoxia, but the

increases of message and protein are similar at rest in hypoxia, but the change in protein is somewhat less than in message during exercise. Whether this could reflect having reached a maximal rate of protein synthesis during exercise cannot be determined from these results.

There is more evidence that intracellular hypoxia drives VEGF responses in skeletal muscle. In a group of patients with chronic renal failure, we assessed both intracellular PO_2 during exercise (by proton MRS) and the VEGF mRNA response to this exercise. The patients were all young and relatively active, without complications of renal disease such as hypertension or other common diseases such as heart disease or diabetes. They were all on regular hemodialysis awaiting renal transplantation, and had been treated with erythropoietin to combat anemia. Figure 11 shows the rest (R) and post-exercise (E) VEGF mRNA levels by Northern blot of 4 control and 4 renal failure patients (top panel) and the relationship between the fold response in VEGF mRNA to exercise and the intracellular PO_2 in the lower panel (35). VEGF message levels were greatly increased in both groups, and

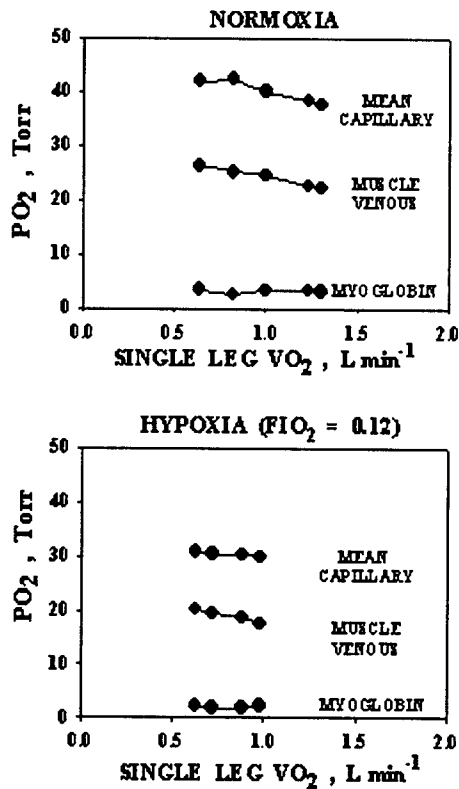


Figure 7. Proton MRS-based estimates of intracellular PO_2 in knee extensor muscle from measurements of myoglobin O_2 saturation in man during exercise in normoxia (upper panel) and hypoxia (lower panel). Also shown are measured femoral vein and calculated mean muscle capillary PO_2 values under the same conditions. Intracellular PO_2 is only 2-3 Torr when vascular PO_2 is 20-30 Torr, implying a large resistance to O_2 transport by diffusion between the capillary and the myocyte cell wall.

appear to relate to degree of hypoxia. Extrapolation to the origin (i.e., to absence of hypoxia) suggests the response would not occur, or at least would be much less, without intracellular hypoxia.

VEGF REGULATION BY NITRIC OXIDE (NO)

An attractive candidate for VEGF regulation is NO. The logic follows from the fact that increased blood flow (and thus O₂ delivery) is critical to muscle function during exercise. This requires local muscle vasodilatation, and NO is known to be released (24) during exercise and contribute to this effect. Hypoxia causes further muscle vasodilatation compared to normoxia, and NO is a small, diffusible molecule that could reach muscle cell nuclei and which is known to act as a transcriptional activator (1). Thus NO, released by the muscle microvascular endothelium consequent to increased blood flow during exercise, can be proposed as a possible messenger activating VEGF transcription in the muscle cell.

We tested that hypothesis three ways. First we gave nitroprusside and acetylcholine (each to different rats) in doses sufficient to increase local muscle vascular conductance about 4-fold and measured the muscle VEGF mRNA response to this increased availability of NO (3). These rats were anesthetized, and so no exercise was performed. Next we inhibited NO synthase acutely with 300 mg IP L-NAME and measured the resting and post-exercise VEGF mRNA levels in treated rats compared to D-NAME treated controls (10). Third, we treated rats for 6 weeks with L-NAME (10 mg/kg in the drinking water) and then examined muscle capillarity morphometrically (16). Figure 12 shows that NO donors augment VEGF mRNA levels (top panel), that NOS inhibition reduces the exercise response of VEGF mRNA (middle panel), and that after 6 weeks of NOS inhibition, muscle capillarity is reduced (lower panel). We suggest, from these three studies taken together, that NO is a biologically significant modulator of VEGF transcription *in vivo*. However, NO appears unlikely to be the sole regulator of VEGF since the effects of NO on mRNA abundance do not match those of exercise, and because L-NAME does not abolish the effect of exercise on VEGF.

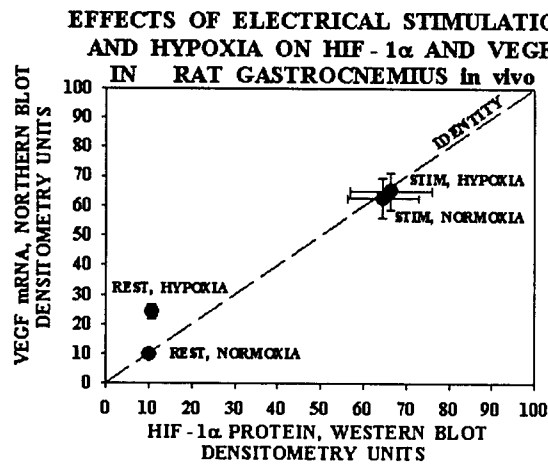


Figure 8. Effects of exercise (in normoxia and hypoxia) and of hypoxia alone ($FI_{O_2} = 0.12$) on both HIF-1 α protein levels and VEGF mRNA levels in rat gastrocnemius. Similar fold changes are seen in both after exercise, while moderate hypoxia alone increases VEGF message without HIF-1 α protein response. See text for more details.

FUTURE DIRECTIONS

The above studies broadly support the hypothesis that intracellular hypoxia is an important stimulus to VEGF gene activation. They are, however, necessarily correlative in nature. Two major directions of study are needed to establish cause and effect, and thus complete the picture. They both must be interventional, and will require gene deletion with subsequent examination of the consequences on physiological outcome. The first of these is to delete the VEGF gene from skeletal muscle and determine the consequences for muscle capillarity. This experiment would show whether VEGF is an essential growth factor without which capillary angiogenesis will not occur. Corresponding studies in tumors in mice show indeed that VEGF is critical to growth of these tumors and their blood supply. The second experiment concerns the role of hypoxia as the stimulus to VEGF. That requires deletion of the HIF-1 α gene from skeletal muscle, with subsequent examination of the VEGF mRNA response to exercise. If hypoxia is key, the VEGF response should be abolished or at least greatly diminished.

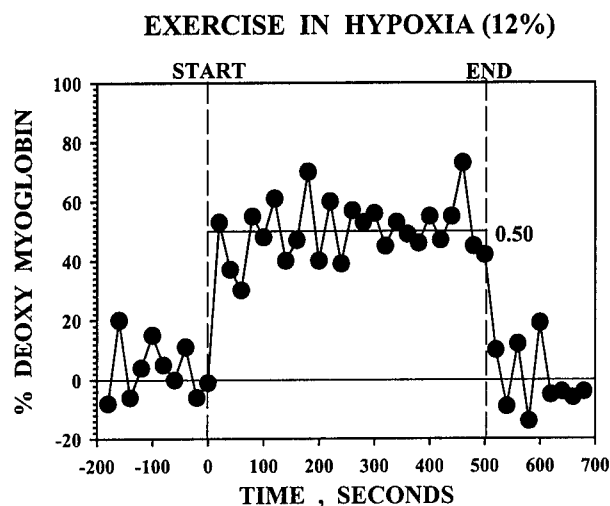


Figure 9. Proton MRS signals reflecting myoglobin deoxygenation gathered over 20 second intervals in a normal subject breathing 12 % O_2 , first at rest (left of left-hand vertical dashed line), then during exercise (between two dashed lines) and finally at rest again (right of right-hand dashed line). Exercise, but not rest, produces myoglobin O_2 desaturation (of about 50% in this example).

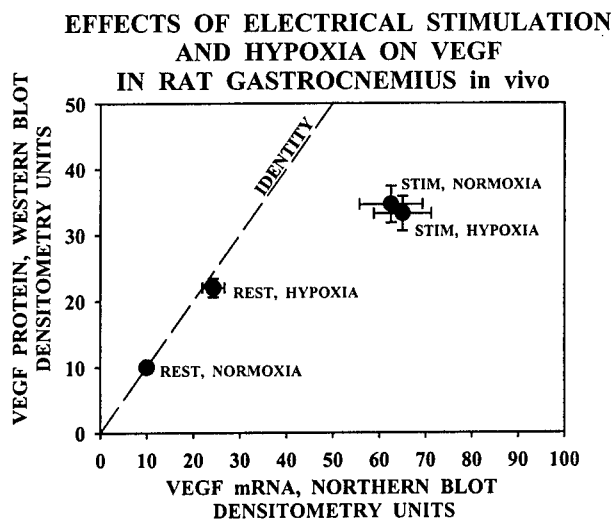


Figure 10. Relationship between VEGF mRNA and protein levels in rat gastrocnemius in response to exercise or hypoxia. There are roughly 2-fold increases in both with hypoxia ($FI_{O_2} = 0.12$) alone, but after exercise, the change in protein, while substantial, does not match that of message. See text for further details.

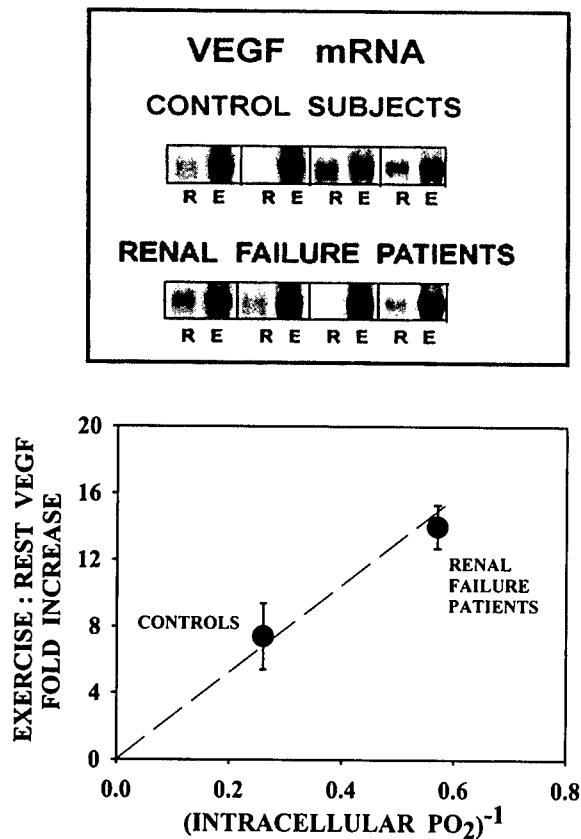
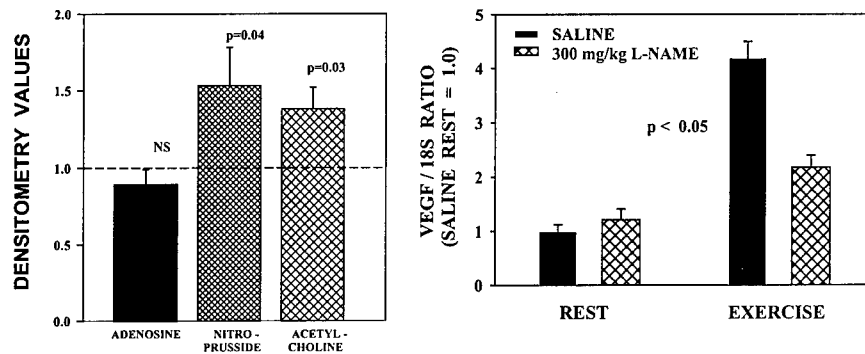


Figure 11. Top panel shows Northern blots for VEGF mRNA from knee extensor biopsies in normal subjects and patients with chronic renal failure. Lanes marked "R" reflect resting biopsies while those marked "E" reflect biopsies done immediately after 45 minutes of knee extensor exercise at about 50% of maximal effort. Both groups show major increases in VEGF mRNA after exercise. Lower panel relates this fold increase in VEGF message to intracellular PO₂ measured in the same subjects by proton MRS. Extrapolation suggests that in the absence of hypoxia the VEGF response would be small or absent.

The problem with such simply stated experiments is that both VEGF and HIF-1 α gene knockouts are embryonically lethal (7,29), such that no mice would survive to test the hypotheses. Even if they did, such long-term studies would be quite susceptible to the development of compensatory mechanisms during embryogenesis and beyond that would cloud the issue. Furthermore, both genes are ubiquitous with more than one effect related to O₂ transport in more than one tissue, making a clean study even more difficult.

Hope exists however in the form of the Cre/LoxP targeted gene deletion method (19). Here, a transgenic mouse is created in which the gene of interest (in the present case, VEGF or HIF-1 α) is modified by including two



EFFECTS OF NOS INHIBITION ON MUSCLE CAPILLARITY

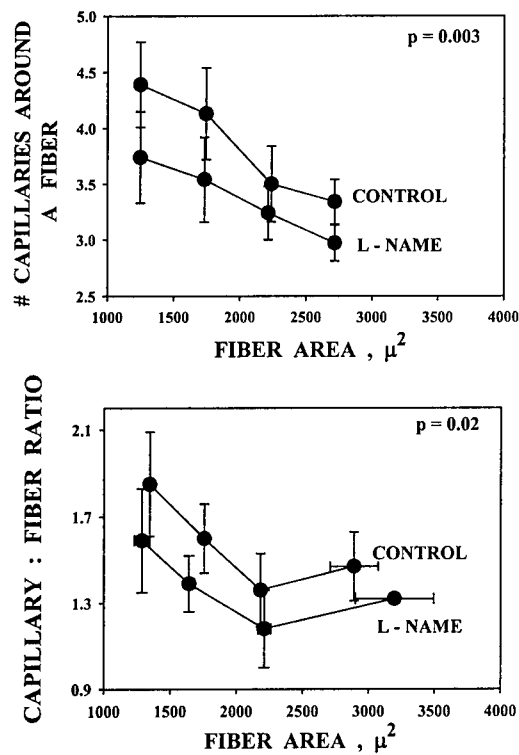


Figure 12. Effects of NO on VEGF mRNA and muscle capillarity. Top left panel shows enhanced message abundance in response to giving nitroprusside or acetylcholine but not another vasodilator (adenosine). Top right panel shows that NOS inhibition acutely with L-NAME reduces, but does not abolish, the VEGF mRNA exercise response. Lower panel shows reduction in muscle capillarity in rats after 6 weeks of imbibing 10 mg/kg L-NAME in the drinking water.

copies of a 34 base pair sequence of non-mammalian DNA referred to as “LoxP”. The copies are inserted into the coding region of the gene to flank important exons. The “floxed” mouse grows to adulthood normally, because the LoxP sequences have no function. When the gene is to be deleted, a viral construct containing DNA encoding the enzyme Cre Recombinase is transfected directly into the “floxed” mouse’s gastrocnemius muscle. The enzyme should then be expressed, but only by the particular muscle cells transfected. Cre Recombinase specifically attacks the two LoxP sequences, thus excising the intervening portion of the VEGF (or HIF-1 α), and recombining the scissored ends of the gene, rendering it non-functional. With appropriate controls for the potential inflammatory effects of the viral vector, the consequences of gene deletion in the locally transfected portion of muscle can be examined. For VEGF deletion, one would check for its message absence by *in situ* hybridization and for its protein absence immunohistochemically. Then, the critical study would be to relate muscle capillarity to absence of VEGF. For HIF-1 α , its absence must also be checked, and then the critical study would be to determine whether VEGF transcription fails to respond to exercise in the HIF-1 α deficient muscle under conditions where it normally would respond. These studies are under way.

ACKNOWLEDGMENTS

The many individual studies summarized herein would not have been carried out without the major involvement of the following co-investigators: Donald E. Bebout, Henri Benoit, Ellen C. Breen, Timothy Gavin, Russell Hepple, Sachiko Homma, Odile Mathieu-Costello, Russell S. Richardson, Amy Sung, Kechun Tang and Harrieth Wagner. The bulk of the research was supported by NIH Grant HL17731.

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Chapter 5

Leukocyte-endothelial interactions in environmental hypoxia

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Abstract: Hypoxia induced by reducing inspired P_{O_2} (P_{IO_2}) to 70 Torr, promotes a rapid microvascular response characterized by increased leukocyte rolling and adherence to the venular endothelium, leukocyte emigration to the perivascular space and increased vascular permeability. This appears to be a generalized response since it is observed in venules of the mesentery, cremaster muscle and pial microcirculations. After three weeks of acclimatization to hypoxia (barometric pressure 380 Torr, P_{IO_2} 70 Torr), the initial microvascular response resolves and exposure to even lower P_{IO_2} (50 Torr) fails to elicit a microvascular response. The initial response is accompanied by a reversible increase in the generation of reactive oxygen species (ROS) and is blocked by antioxidants and by interventions that increase the tissue levels of nitric oxide (NO). In contrast to ischemia/reperfusion, ROS levels increase during hypoxia and return towards pre-hypoxic values after return to normoxia. Acclimatization involves upregulation of inducible NO synthase (iNOS): inhibition of iNOS using two different antagonists results in increased leukocyte-endothelial interactions and increased ROS generation. The results suggest that hypoxia initially leads to an alteration of the ROS/NO balance which is eventually restored during the acclimatization process. This phenomenon may have relevance to the microcirculatory alterations associated with hypoxic exposure, including acute mountain sickness and high altitude pulmonary and cerebral edema.

Key words: nitric oxide, reactive oxygen species, microcirculation, nitric oxide synthase, inflammatory response.

INTRODUCTION

Environmental hypoxia is frequently associated with pathophysiological features that are consistent with microvascular injury due to increased leukocyte-endothelial interactions (15, 35). In spite of this evidence, relatively few studies have been directed to examine the effects of environmental hypoxia on this subject. On the other hand, considerable effort has been directed to identify the mechanisms responsible for microvascular injury associated with ischemia/reperfusion (I/R). It is generally accepted that an initiating cause in the microcirculatory alterations in I/R is generation of reactive oxygen species (ROS) upon reintroduction of oxygen after a period of total ischemia (11, 14).

Circulating leukocytes play a key role in the pathogenesis of I/R as well as other conditions such as sepsis and inflammation, through a coordinated process (Figure 1) which first involves leukocyte rolling, followed by firm adherence of leukocytes to venular endothelial cells and emigration to the perivascular space following chemotactic gradients (10, 11). These processes are mediated by specific glycoproteins expressed on the surface of leukocytes and endothelial cells (11, 24, 28). Adherent leukocytes release proteolytic enzymes and ROS which could injure the underlying venular endothelium, and result in increased vascular permeability (10, 14, 19). The relatively little attention attracted by the effect of environmental hypoxia on leukocyte-endothelial interactions is rather surprising in light of several lines of evidence suggesting that vascular endothelial function may be altered in conditions of hypoxia not associated with ischemia:

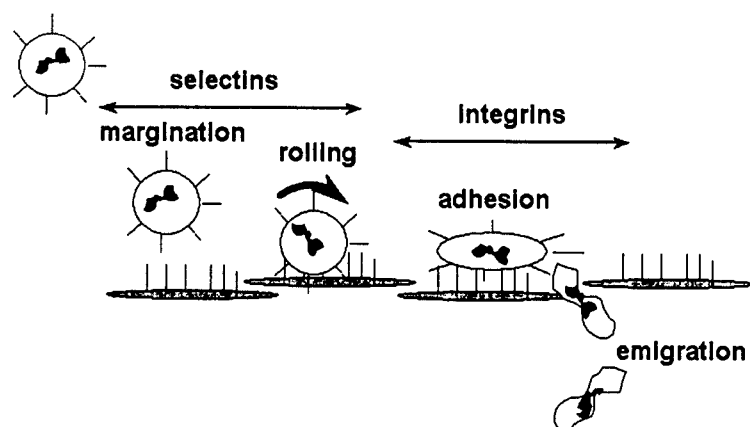


Figure 1. Schematic representation of leukocyte-endothelial interactions leading to leukocyte adherence and emigration.

1. Altitude hypoxia may result in high altitude pulmonary (HAPE) and cerebral edema (HACE)

The relative contribution of endothelial damage and mechanical vessel disruption in the pathogenesis of HAPE has not been clearly elucidated (15); however, endothelial damage is suggested by elevated plasma levels of E-selectin (13) and by the presence of leukocytes and inflammatory mediators in broncho alveolar lavage fluid (20) and in the urine (17) of patients with HAPE. With respect to the pathogenesis of HACE, a key role of microcirculatory alterations has been proposed based on the observation of increased brain angiogenesis during hypoxia (22). This latter response is related to upregulation of vascular endothelial growth factor (VEGF), the levels of which are increased in rats exposed to severe hypoxia (40). VEGF-mediated angiogenesis involves an initial period of increased vascular permeability (26) which could contribute to the development of HACE.

2. ROS are generated during hypoxia

Reduced Po_2 , in the absence of ischemia, can result in the generation of ROS in isolated cardiomyocytes as well as other cell types (7, 30). In addition, hypoxia has been shown to reduce the levels of endogenous antioxidants in cultured endothelial cells (31) as well as in the liver *in vivo* (8), which was attributed to hypoxia-induced ROS formation. Recent observations indicate the mitochondria as the source of ROS generated during reductions of Po_2 (5).

3. Nitric Oxide (NO) levels are reduced during hypoxia

NO is an anti-inflammatory mediator which prevents leukocyte-endothelial adhesive interactions (10, 24, 28). Hypoxia decreases NO formation in endothelial cells *in vitro* (37) and in isolated-perfused lungs (18), suggesting a potential role for NO depletion in the microvascular response to environmental hypoxia.

4. In vitro studies show increased leukocyte-endothelial interactions during hypoxia

A reduction in Po_2 , in the absence of other changes in the medium composition, promotes leukocyte adherence to cultured endothelial cells (1), incubated umbilical vein cells (3), and endocardial cells (9). These data suggest that reduced Po_2 can initiate changes that may lead to microvascular damage.

5. ROS generation increases expression of hypoxia inducible factor -1 (HIF-1)

Intracellular ROS generation has been proposed as one of the promoters of HIF-1 (6, 36), a nuclear protein expressed in endothelial cells that activates gene transcription, resulting in cellular changes that include expression of inducible NO synthase (iNOS), heme oxygenase 1 (HO-1) and VEGF (16, 34). iNOS upregulation in the pulmonary vasculature during chronic hypoxia has been demonstrated recently (23, 27). During degradation of heme, HO-1 forms carbon monoxide (CO), a potent vasodilator with anti-inflammatory properties similar to NO (25, 29). Recently, increased iNOS expression in endothelial cells was shown to increase cellular levels of HO-1 (33). Accordingly, it is possible that iNOS upregulation may represent an adaptation to prolonged hypoxia.

In summary, several lines of evidence suggest that a reduction in P_{O_2} may result in significant modifications in vascular endothelial function. However, most of this evidence has been obtained in *in vitro* systems, and the relevance of these findings to the intact animal is sometimes difficult to ascertain. What follows is a description of our own studies on the effect of environmental hypoxia on leukocyte-endothelial interactions in the microcirculation of intact animals.

Effect of hypoxia on the vascular endothelial function of intact animals

Using the technique of intravital microscopy, we have observed that systemic hypoxia, induced by breathing 10% O_2 , ($P_{IO_2} \sim 70$ Torr) results in a rapid increase in leukocyte-endothelial adhesive interactions of mesenteric venules of intact rats (39). This response is characterized by an increase in the number of rolling leukocytes (i.e., leukocytes moving along the venular endothelium at a rate lower than red blood cell velocity), followed by firm leukocyte adherence to the endothelium. This is a very rapid response (Figure 2) with leukocyte adherence increasing significantly above normoxic values by 4 min of hypoxia. Shear rate—the force generated at the vascular wall by the movement of blood—decreases during hypoxia as a result of sympathetic-mediated mesentery vasoconstriction (21), and this may tend to increase leukocyte adherence, although we will show that this is not a major contributing factor in this phenomenon. Upon return to normoxia, shear rate increases above pre-hypoxic values. Although leukocyte adherence tends to decrease during recovery, it remains substantially elevated above baseline, indicating firm adhesive interactions.

Studies of the microcirculation of rats which had been exposed to hypoxia in the conscious state for 2 and 4 h show significant emigration of

leukocytes to the perivascular space (Figure 3), as well as a significant increase in microvascular permeability as indicated by extravasation of fluorescence-labeled albumin (Figure 4). While this response was initially observed in the mesenteric microcirculation (Figure 5), similar findings in the cremaster (Figure 6) and the pial microcirculation indicate that this is a generalized response to a reduction in P_{O_2} .

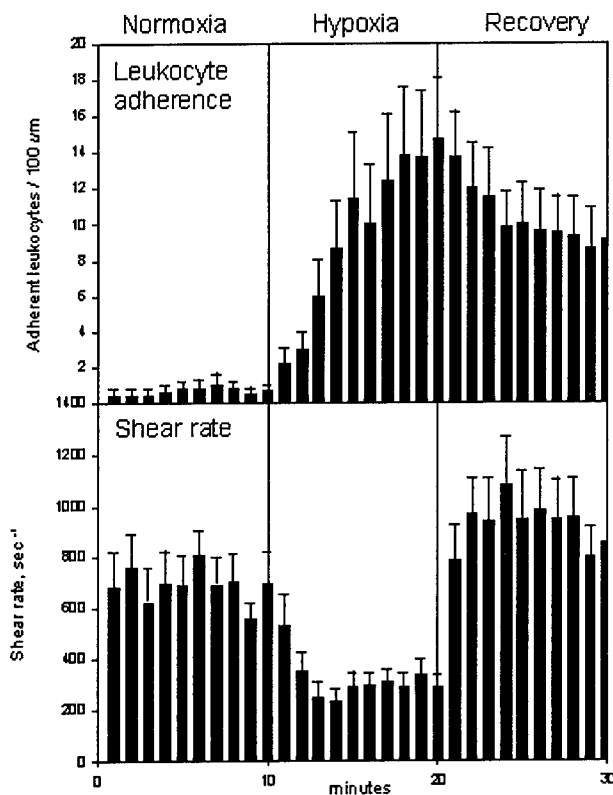


Figure 2. Effect of acute systemic hypoxia (P_{aO_2} 37.6 ± 1.4 Torr) on leukocyte adherence (top panel) and shear rate (bottom panel) in venules of the mesenteric circulation. Data are mean \pm SEM of 6 animals.

After 3 weeks of acclimatization to a barometric pressure of ~ 380 Torr, which also results in a P_{IO_2} of ~ 70 Torr, the animals show no evidence of microvascular lesion in either the mesentery or the cremaster, and no emigrated leukocytes are observed in the perivascular space (Figure 7). Furthermore, exposing the animals to an inspired P_{O_2} of 50 Torr -which results in arterial P_{O_2} levels of less than 30 Torr- or lowering the hemoglobin concentration of these acclimatized rats to normal values while breathing 10% O_2 , does not stimulate leukocyte-endothelial adhesive interactions in the mesenteric circulation (39).

These data show that hypoxia produces a substantial response in vascular endothelial function which takes place in several microcirculatory beds, suggesting a generalized phenomenon. Prolonged exposure to hypoxia results in a resolution of the microvascular injury and an increased resistance

of the vascular endothelium to hypoxia, manifested by a lack of effect of further reductions in P_{O_2} or blood O_2 content.

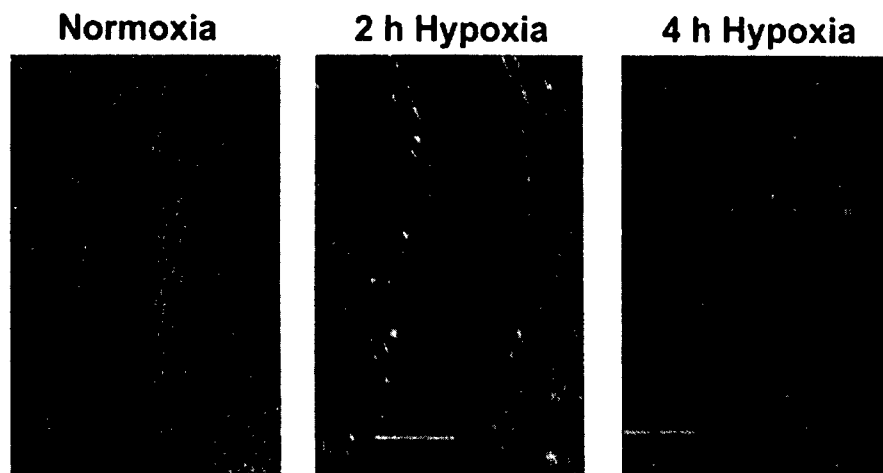


Figure 3. Leukocyte emigration in the mesenteric circulation of rats which were exposed in the conscious state to room air (left panel), and 10 % O_2 for 2 h (middle panel) or 4 h (right panel). The large black dots are used to align the optical Doppler velocimeter to obtain red blood cell velocity. After measurement, the dots are sometimes placed outside the vessel to visualize a larger length of vessel wall. After 2 h of hypoxia, several leukocytes can be observed outside the vessel close to the left side of the venule. After 4 h, a larger number of leukocytes which have emigrated farther away from the venule can be observed. Average number of emigrated leukocytes observed in an area $4 \times 10^3 \mu m^2$ are: normoxia ($n=11$) 1.8 ± 0.8 ; 2h hypoxia ($n=11$) 6.1 ± 1.3 ; 4 h hypoxia ($n=10$) 11.5 ± 1.6 . These results show that hypoxia-induced leukocyte adherence and emigration occur in the physiological setting of the intact, conscious animal.

THE ROS/NO BALANCE AND THE MICROVASCULAR RESPONSE TO HYPOXIA

As indicated above, several lines of evidence show that ROS are generated during hypoxia. ROS could mediate the vascular endothelial response to hypoxia through various mechanisms. One could involve expression of the adhesion molecules that mediate leukocyte rolling and adherence (12). ROS may also produce damage to cell membranes resulting in the release of lipid inflammatory mediators from leukocytes, endothelial, and mast cells (12, 32). These mediators, in turn, can contribute to the microvascular response to hypoxia. Additionally, ROS may interact with NO to form peroxynitrite and other potentially toxic reactive nitrogen oxide species (2). Depletion of the anti-inflammatory agent NO by ROS may by itself contribute to the hypoxic response. A role of changes in the ROS/NO

balance in the microcirculatory response to hypoxia is suggested by the following observations:

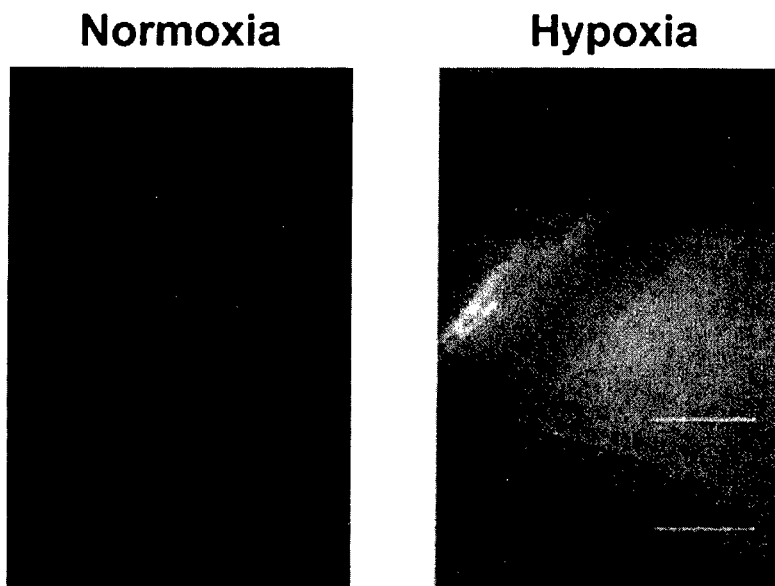


Figure 4. FITC-albumin fluorescence of a mesenteric venule of a rat exposed to normoxia (left panel) and of a different rat exposed in the conscious state to hypoxia for 4 h (right panel). FITC-albumin was injected 30 min before the animals were anesthetized and rapidly prepared for intravascular microscopy. In the normoxic animal, albumin fluorescence is confined to the intravascular space. In the hypoxic animal, fluorescent albumin is present in the extravascular space indicating an increase in vascular permeability. The ratio of perivascular to intravascular fluorescence intensity was: normoxia (n=4) 0.12 ± 0.03 , hypoxia (n=7) 0.99 ± 0.07 .

a. ROS are generated in the mesenteric and cremasteric microcirculation during hypoxia

Dihydrorhodamine 123 (DHR), an oxidant sensitive probe, was used to determine ROS generation in the microcirculation. Oxidation of DHR, primarily by hydrogen peroxide-dependent reactions, forms rhodamine 123, which fluoresces. Rhodamine 123 binds to the inner mitochondrial membrane which is a major site of ROS generation in hypoxia (4). Fig 8 shows photographs of a mesenteric venule demonstrating that rhodamine

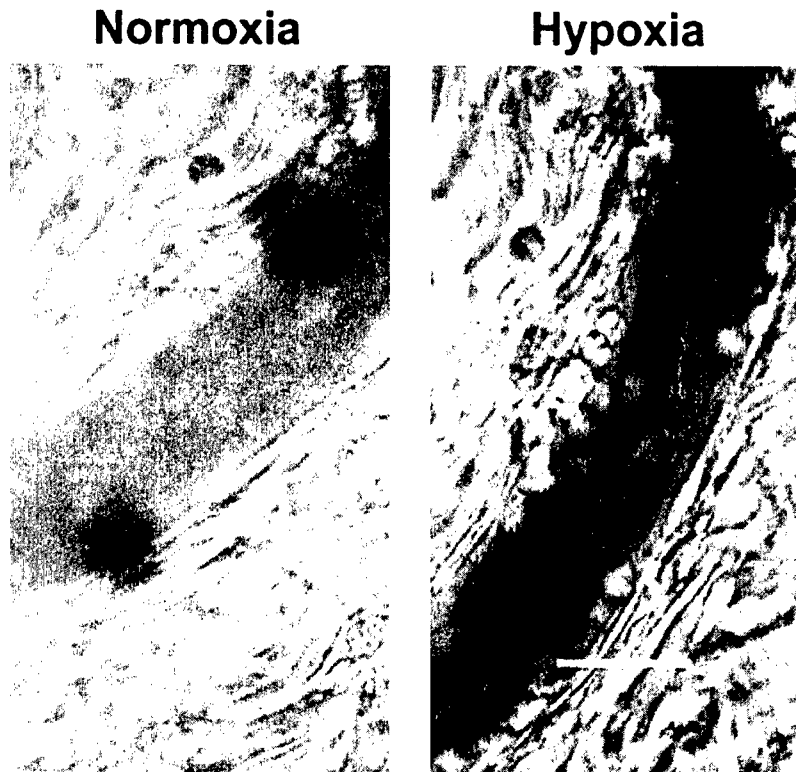


Figure 5. Photograph of a mesenteric venule of a rat exposed to room air (left panel) and after 10 minutes of breathing 10 % O₂ (right panel). Virtually no leukocytes can be observed adhering to the vessel wall during normoxia. In contrast, a large number of adherent leukocytes are present after only 10 min of hypoxia.

fluorescence intensity during the normoxic control period is low (left panel) but increases significantly at 10 min of hypoxia (middle panel) and decreases towards baseline levels after return to normoxia (right panel). Fluorescence is particularly noticeable on the edges of the vessels, which are the sites of leukocyte adherence to the endothelium.

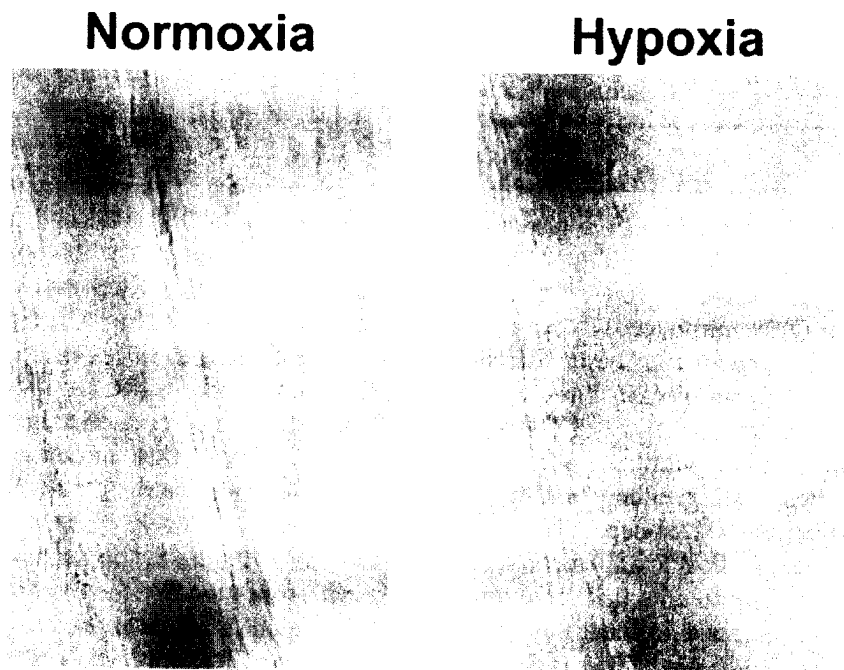


Figure 6. Photograph of a cremasteric venule of a rat breathing room air and after 10 min of 10% O₂ breathing. As in the mesentery, cremaster venules are virtually devoid of adherent leukocytes in normoxia, with leukocyte adherence increasing dramatically after 10 min of hypoxia.

Table 1 shows the cumulative results of 6 rats in which fluorescence intensity in the mesentery is calculated using image analysis (NIH Image 1.61) and expressed as percent of the normoxic control period. Fluorescence intensity increases during hypoxia and returns to control values in the normoxic recovery period. The effect of hypoxia on the intensity of the fluorescence signal is abolished by pretreatment with the antioxidants superoxide dismutase (SOD)/catalase, indicating that fluorescence levels are indeed representative of tissue ROS levels (Table 1). Similar changes in DHR fluorescence intensity in response to hypoxia and antioxidant treatment are observed in the cremaster, showing that hypoxia induces a reversible increase in ROS levels in this microcirculatory bed as well as in the mesentery.

Table 1. Effect of antioxidant treatment on ROS-dependent fluorescence intensity in non-acclimatized rats.

Normoxia	Hypoxia	Recovery	Normoxia + SOD/catalase	Hypoxia + SOD/catalase	Recovery + SOD/catalase
100	272 ±41	95 ±24	100	88.1 ±7.5	92 ±10

The pattern of ROS generation in hypoxia is clearly distinct from that observed in I/R (38). In hypoxia, ROS levels increase rapidly after the onset of hypoxia, and decrease upon return to normoxia. In I/R, ROS increase when O₂ is introduced after a prolonged ischemic period, during which no ROS are generated. These results indicate fundamental differences between the mechanisms responsible for ROS generation in hypoxia vs I/R.

A central role for ROS in hypoxia-induced leukocyte-endothelial interactions is further supported by the observation that antioxidant treatment (using either SOD/catalase or lipoic acid) not only blocks the increase in oxidant-dependent fluorescence but also completely inhibits the increased leukocyte adherence that follows hypoxia (Figure 9, top panel). It is interesting to note that antioxidant treatment did not modify the hypoxia-induced decrease in shear rate (Figure 9, bottom panel), which suggests that this factor did not play a major role in the increased leukocyte-endothelial interactions.

Antioxidant treatment also significantly attenuated the increases in leukocyte emigration (Fig 10, top panel) as well as in vascular permeability (Figure 10, bottom panel) observed after conscious rats were exposed to 4 h of hypoxia. Taken together, these results indicate that hypoxia is accompanied by an increase in ROS generation, and that the ROS are important determinants of the microvascular response to acute hypoxia.

b. Increasing tissue NO levels significantly attenuates the microvascular response to hypoxia.

Fig 11 shows the effects of increasing tissue NO levels, induced by superfusing the mesentery with either spermine NONOate, a NO donor, or with the NO precursor, L- arginine. Both treatments significantly reduced the adherence of leukocytes to the venular endothelium during hypoxia. This effect was not due to a larger venular shear rate which was similar in untreated and treated groups. NO donor treatment also resulted in attenuation of the increase in microvascular permeability (Figure 12) and leukocyte emigration (Figure 13) observed when conscious rats were maintained in hypoxia for 4 h.

While it is possible that the effects of NO on the microvascular response to hypoxia reflect the anti-inflammatory properties of NO, it is also possible that the effect of hypoxia may be mediated through a decrease in NO levels

due to inactivation of NO by ROS to form peroxynitrite. This second possibility is supported by the observation that, in addition to attenuating the increase in leukocyte-endothelial interactions, pretreatment with NO donors prevents the increase in ROS generation induced by hypoxia (Table 2).

In summary, our studies show that acute systemic hypoxia elicits a vascular endothelial response characterized by leukocyte rolling and adherence to the venular endothelium, increased vascular permeability, and leukocyte emigration to the perivascular space. This response is observed in the mesentery, cremaster, and pial microcirculations, and occurs in both anesthetized and conscious rats. A key pathophysiological feature is an alteration of the ROS/NO balance; restoration of this balance substantially attenuates or eliminates all of the features of the response to hypoxia. Several important factors remain unknown, including the source and mechanisms of ROS generation, and the possible participation of other factors such as lipid inflammatory mediators and cytokines, both of which are known to mediate leukocyte-endothelial interactions in other conditions such as ischemia/reperfusion, sepsis and inflammation.

Table 2. Effect of administration of an NO donor on ROS-dependent fluorescence intensity in non-acclimatized rats.

Normoxia	Hypoxia	Recovery	Normoxia + NO donor	Hypoxia + NO donor	Recovery + NO donor
100	166.3 ±17.9	112 ±10.9	100	101.2 ±5.9	92.4 ±5.6

Acclimatization of the venular endothelium to prolonged hypoxia

If hypoxia is maintained, the strong initial microvascular response eventually resolves. After three weeks of acclimatization, no evidence of leukocyte adherence and emigration, or of increased vascular permeability, are observed in either mesentery or cremaster (Figure 7). The mechanisms responsible for the acclimatization of vascular endothelial function are not clear. A key feature of acclimatization is an increase in red blood cell mass response to increased erythropoietin secretion. This factor could have contributed to the endothelial acclimatization by two mechanisms: on one hand, the elevated hematocrit increases blood viscosity and tends to increase shear rate, which, in turn, opposes the leukocyte-endothelial adhesive forces. Secondly, the elevated hemoglobin concentration may improve oxygen delivery and attenuate the severity of hypoxia at the venular level. While these factors may participate in the acclimatization of vascular endothelial function, they probably have only a secondary role. As indicated above, leukocytes remain adherent to the endothelium during the normoxic

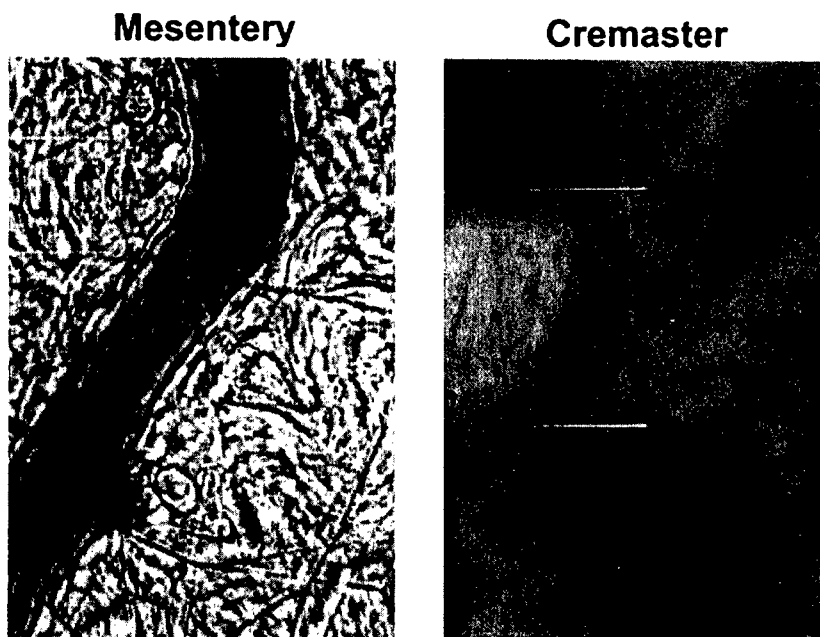


Figure 7. Photograph of a mesenteric venule (left panel) and a cremasteric venule (right panel) of rats acclimatized to hypoxia for three weeks. Although both animals breathe 7.5 % O_2 ($P_{IO_2} \sim 50$ Torr) no leukocyte adherence is observed in either microcirculation.

recovery after acute hypoxia, in spite of a return to control shear rate values, indicating that the adhesive forces are strong enough to oppose normal shear rate levels. Secondly, further reductions of the inspired P_{O_2} to attain arterial blood P_{O_2} levels as low as 28 Torr, or decreasing Hb concentration to normal values, do not increase leukocyte adherence to the endothelium of mesenteric venules of acclimatized rats. Since the initial response to hypoxia is linked to an increase in the ROS/ NO balance, it seems plausible that the modifications in vascular endothelial function observed during acclimatization may be associated with a restoration of this balance. This possibility is supported by two observations: ROS levels do not change when acclimatized rats are exposed to hypoxia, and inhibition of inducible NO synthase (iNOS) results in an increase in leukocyte rolling and adherence, and in ROS generation, during hypoxia in acclimatized rats.

Since the initial response to hypoxia is linked to an increase in the ROS/ NO balance, it seems plausible that the modifications in vascular endothelial function observed during acclimatization may be associated with a restoration of this balance. This possibility is supported by two observations: ROS levels do not change when acclimatized rats are exposed to hypoxia, and inhibition of inducible NO synthase (iNOS) results in an increase in

leukocyte rolling and adherence, and in ROS generation, during hypoxia in acclimatized rats.

Figure 14 shows that ROS-dependent fluorescence intensity in the mesenteric microcirculation of an acclimatized rat is not affected by changes in P_{O_2} . On the other hand, iNOS inhibition using the iNOS blocker L-NIL results in an increase in ROS-dependent fluorescence when acclimatized rats are exposed to hypoxia. Recent studies in our laboratory show that iNOS inhibition has a similar effect on ROS levels in the cremaster muscle microcirculation of hypoxia-acclimatized rats.

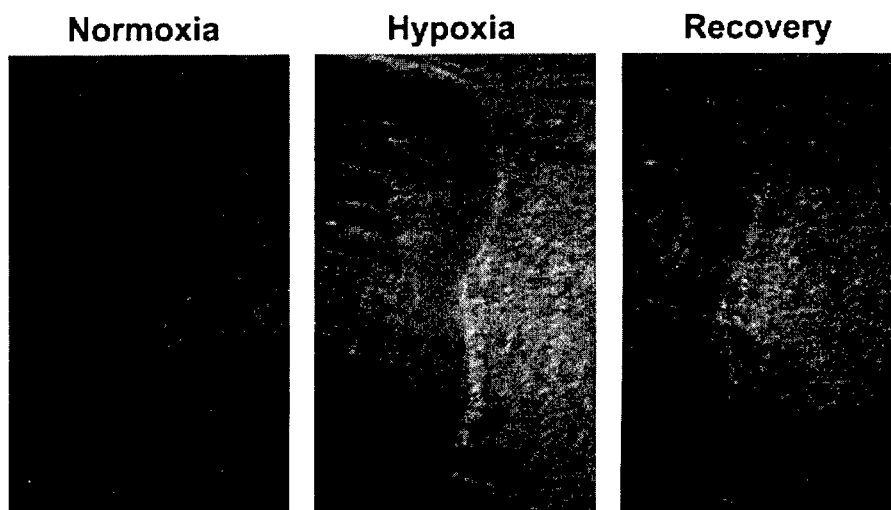


Figure 8. Photograph of a mesenteric venule showing DHR-dependent fluorescence. During the normoxic control (left panel) fluorescence levels are very low, and increase markedly after 10 min of hypoxia (middle panel). Notice that the highest fluorescence intensity occurs at the vessel wall. The right panel photograph was obtained after 10 min of normoxic recovery and shows that fluorescence has decreased toward control levels.

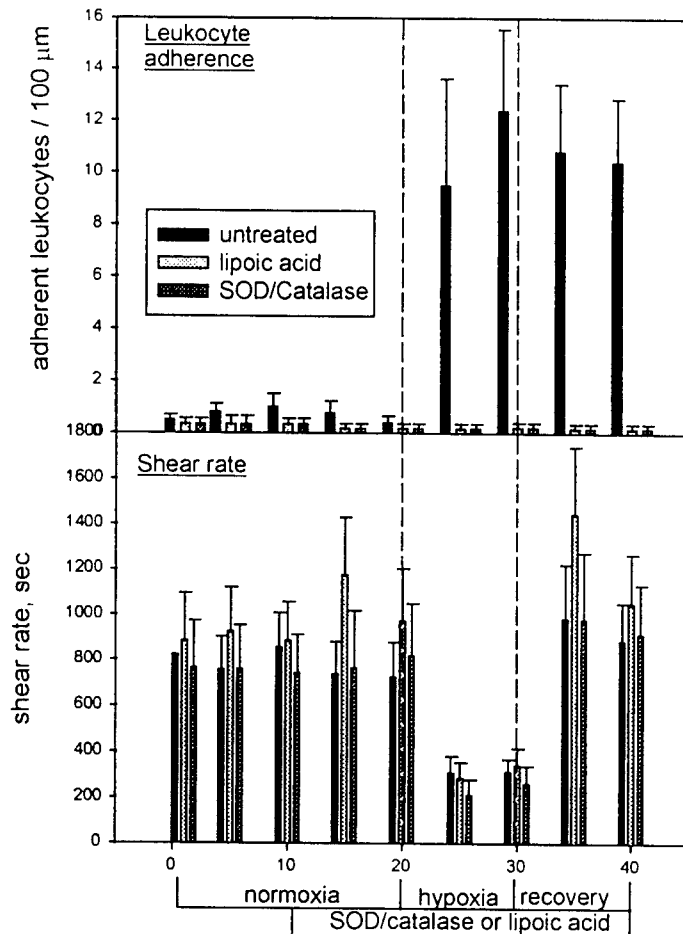


Figure 9. Effect of antioxidant treatment with SOD/catalase or lipoic acid on hypoxia-induced leukocyte adherence (top panel) and shear rate (lower panel). Treatment with either antioxidant completely blocks leukocyte adherence during hypoxia without modifying the effects of hypoxia on shear rate.

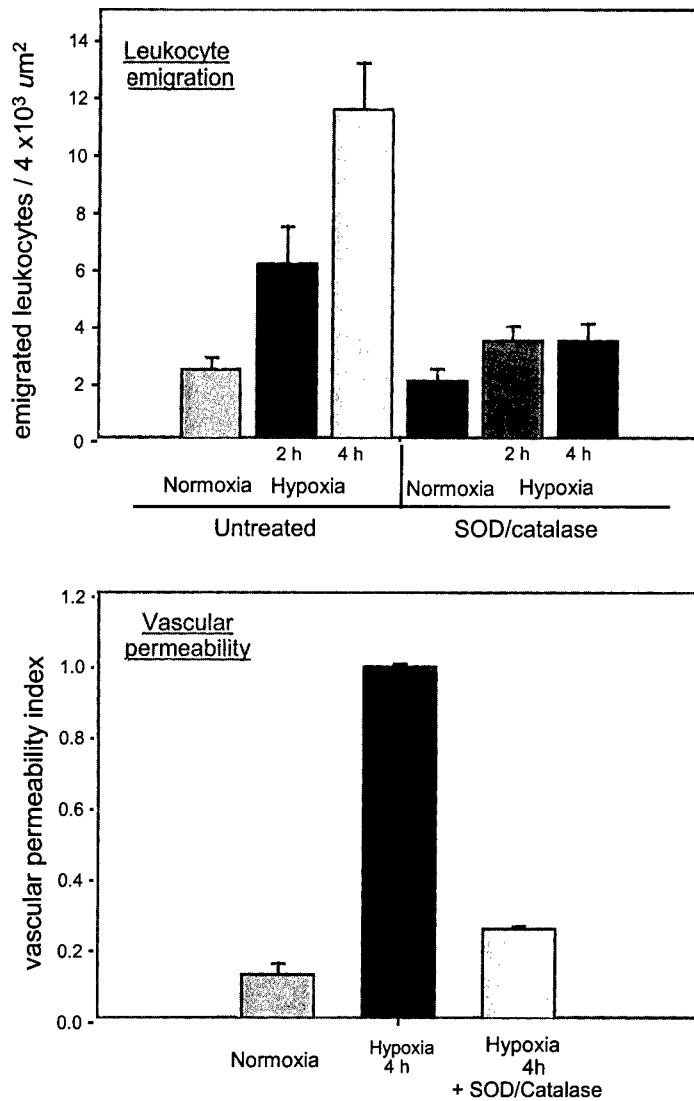


Figure 10. Effect of treatment with SOD/catalase on leukocyte emigration (top panel) and vascular permeability (lower panel) of the mesenteric circulation of conscious rats exposed to normoxia and 2 or 4 h of hypoxia. Leukocyte emigration is evaluated by the number of emigrated leukocytes in an area of $4 \times 10^3 \mu m^2$. Vascular permeability is evaluated by the ratio of perivascular -to-intravascular FITC-albumin fluorescence intensity.

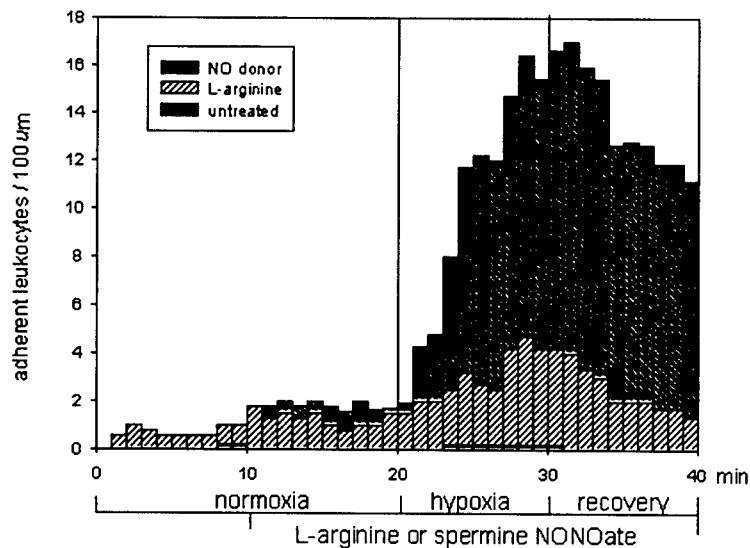


Figure 11. Effect of treatment with the NO precursor L-arginine or the NO donor spermine NONOate on hypoxia-induced leukocyte adherence in the mesenteric microcirculation. Spermine NONOate completely abolished the leukocyte adherence secondary to hypoxia, while L-arginine decreased it significantly.

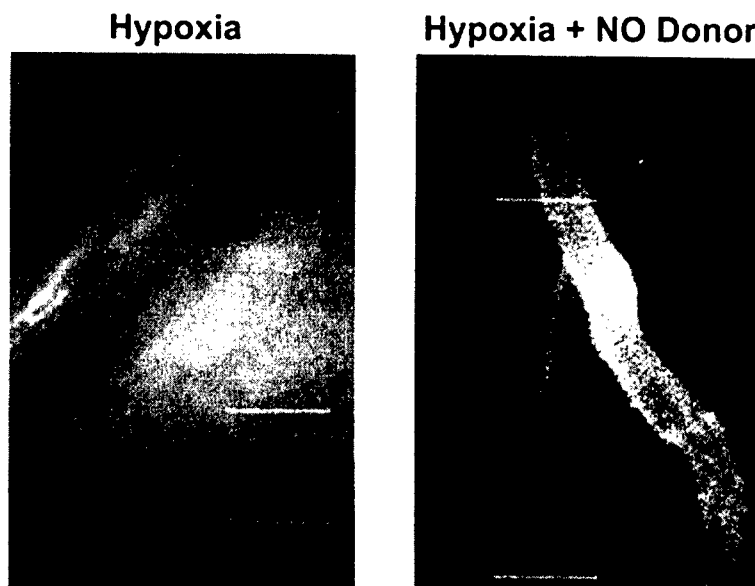


Figure 12. Effect of continuous i.v infusion of spermine NONOate on FITC albumin fluorescence intensity of the mesenteric microcirculation of conscious rats exposed to 10% O₂ for 4 h. FITC albumin was injected 30 min before the animals were anesthetized to observe the microcirculation. The left panel illustrates the increase in vascular permeability during hypoxia, as evidenced by the high perivascular fluorescence intensity. This effect is greatly attenuated by treatment with the NO donor (right panel).

The effect of iNOS inhibition on hypoxia-induced ROS generation in acclimatized rats is associated with increased leukocyte-endothelial adhesive interactions in the mesenteric circulation (Figure 15). While leukocyte adherence is not modified by iNOS inhibition in normoxia, it increases significantly after 10 min of hypoxia. However, the increase in leukocyte adherence promoted by iNOS inhibition during hypoxia is significantly lower in the acclimatized rats than in the non-treated, non-acclimatized rats. This difference could point to additional mechanisms, besides increased iNOS activity, on the process of acclimatization of the vascular endothelium. A potential non-specific effect of 1, 4 PBIT, was ruled out by the observation that this agent had no demonstrable microcirculatory effects in normoxic, non-acclimatized rats, in which iNOS should not be present in significant amounts. Furthermore, similar results were obtained using the structurally different iNOS inhibitor L-NIL.

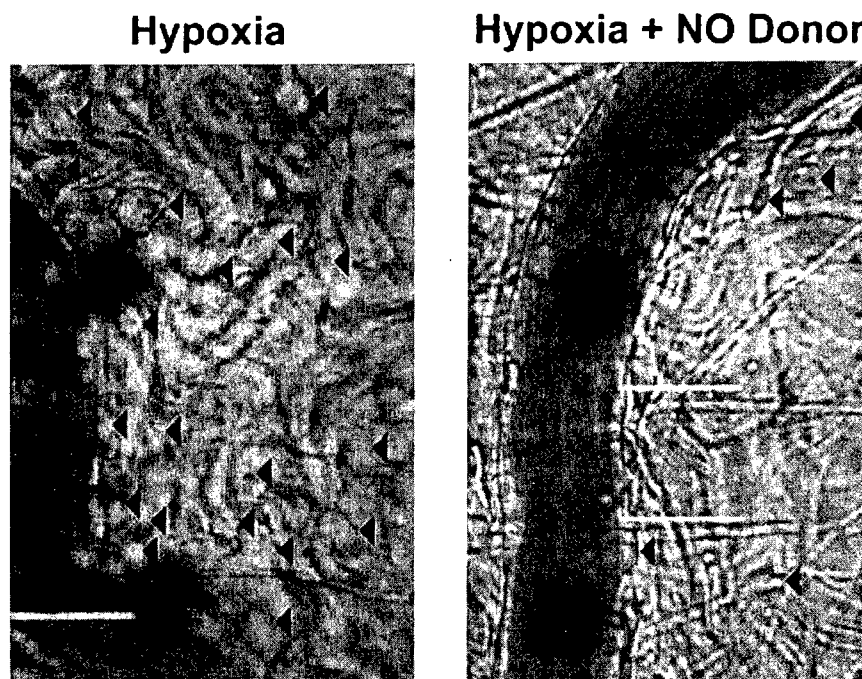


Figure 13. Effect of continuous i.v. infusion of spermine NONOate on leukocyte emigration in the mesenteric microcirculation of rats exposed to 10% O₂ for 4 h. Treatment with the NO donor (right panel) substantially attenuates the hypoxia-induced emigration of leukocytes.

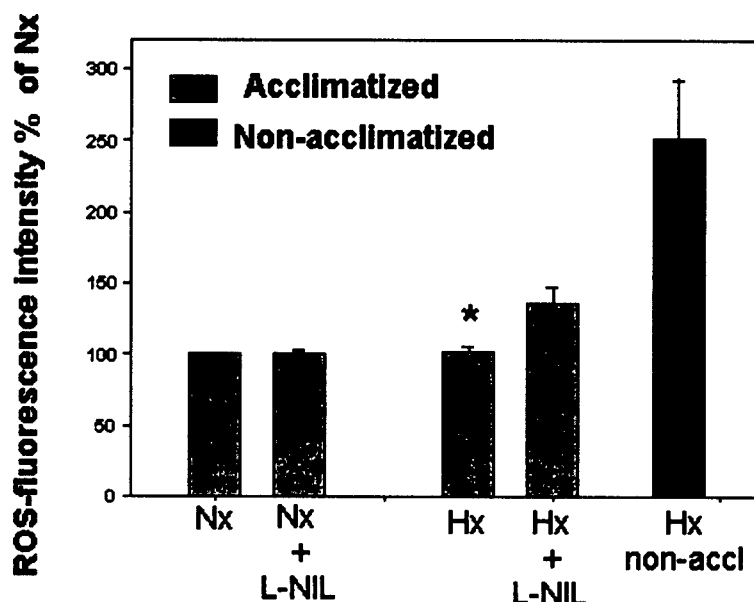


Figure 14. Effect of inhibition of iNOS with the antagonist L-NIL. Nx, acclimatized rats breathing a normoxic gas mixture. Hx, acclimatized rats breathing a hypoxic gas mixture. Hx non-accl: non acclimatized rats breathing a hypoxic gas mixture. ROS fluorescence intensity is expressed as percent of the normoxic, untreated control (Nx).

The effects of iNOS inhibition on leukocyte-endothelial interactions and ROS levels suggest that the process of acclimatization results in upregulation of iNOS. Under normal conditions, NO is formed by eNOS, an enzyme that is constitutively expressed in endothelial cells. However, the inducible form can be expressed in response to various agents. Since iNOS is capable of generating larger amounts of NO than eNOS, our data suggest that NO levels in the vascular endothelium of acclimatized rats are elevated. As demonstrated in the non-acclimatized rats, increased NO levels inhibit leukocyte-endothelial interactions and may be responsible in part for the effect of acclimatization on vascular endothelial function.

The upregulation of iNOS may be secondary to induction of HIF-1. In this respect, it is interesting to note that it has been shown recently that intracellular ROS generation is one of the promoters of HIF-1 (6, 36). HIF-1 activates gene transcription and results in multiple cellular changes, including increased expression of iNOS, VEGF and HO-1. Accordingly, it is possible that the increased ROS generation may

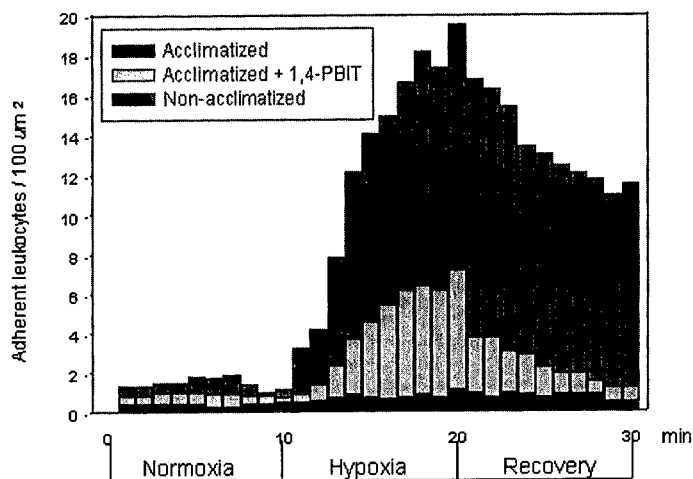


Figure 15. Leukocyte adherence during hypoxia in non-acclimatized rats, and in untreated acclimatized rats and acclimatized rats treated with the iNOS inhibitor 1, 4-PBIT. Leukocyte adherence increases rapidly after hypoxia in the non-acclimatized rats, while it remains at baseline levels in the untreated acclimatized rats. iNOS inhibition in the acclimatized rats using 1, 4 -PBIT results in a significant increase in leukocyte adherence during hypoxia; however, leukocyte adherence does not reach the levels observed in the non-acclimatized rats.

participate, at least in part, in initiating the cascade of events that eventually leads to acclimatization of the vascular endothelium. This would be consistent with the emerging concept that ROS not only result in microvascular injury, but under some circumstances may play a role as signaling elements in the microvascular response to hypoxia.

In summary, exposure to environmental hypoxia results in increased-leukocyte endothelial interactions characterized by increased leukocyte rolling and adherence to the vascular endothelium, followed by leukocyte emigration and increased vascular permeability. This response is observed in several microcirculatory beds suggesting a generalized response to hypoxia. After three weeks of acclimatization, the inflammatory response resolves and the vascular endothelium is resistant to lower P_{O_2} levels. A key pathogenic feature appears to be an alteration in the ROS/NO balance. The initial response is characterized by a change in the ROS/NO balance in favor of the former, while the acclimatization process features a restoration of this balance, in part thanks to upregulation of iNOS. Several questions remain unanswered, which include the nature and mechanism of production of the ROS involved, the interactions between leukocytes and endothelial cells as well as other cell types such as mast cells, and the possible participation of other factors. This includes participation of inflammatory mediators and

cytokines in the initial phase of the response, and of endogenous antioxidants, HIF-1 and associated factors in the acclimatization phase.

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Chapter 6

Hypoxia training for sea-level performance

Training high - living low

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Abstract: It is widely accepted that prolonged exposure to extreme altitude is detrimental for exercise performance and muscle structure. Moreover, highly trained subjects seem to suffer more under hypoxic conditions than untrained people. When using hypoxia as an ergogenic stimulus in athletes, it has thus become customary to limit hypoxia exposure in terms of altitude and duration of exposure in order to achieve defined physiologic goals. If hypoxia application is limited to the duration of training sessions, specific hypoxia responses on the molecular level in skeletal muscle tissue can be demonstrated. Hypoxia inducible factor 1 (HIF-1 α mRNA) is upregulated after 6 weeks of endurance training in hypoxia (equivalent to an altitude of 3850m) in previously untrained subjects. This upregulation is independent of training intensity but not observed in subjects training under similar conditions in normoxia. High intensity training in hypoxia further results in an increase of vascular endothelial growth factor (VEGF) mRNA, capillarity and myoglobin mRNA. These results suggest that hypoxia training results in improvements of the oxygen transfer capacity in skeletal muscle tissue. They thus offer a plausible explanation for the observation that effects of hypoxia training in athletes can best be demonstrated when performance tests are carried out in hypoxia. Beneficial effects of "training high – living low" for sea level performance of athletes can be inferred from the structural changes observed in muscle tissue; however, the functional improvements remain to be demonstrated directly.

Key words: skeletal muscle, mRNA, HIF, VO_2 max, oxidative enzymes, mitochondria, HSP 70

INTRODUCTION

The original observation of altitude being an adaptive stimulus for human skeletal muscle tissue was made by Reynafarje (14) who observed on biopsies of the sartorius muscle that the concentration of oxidative enzymes and of myoglobin was higher in high-altitude natives than in sea-level dwellers. This observation helped to establish the concept of local tissue hypoxia as an important factor for skeletal muscle tissue adaptation to exercise (see 11). It was realized later that exposition of low-landers to severe hypoxia such as during real or simulated ascents to Mt. Everest resulted in a pronounced deterioration of muscle tissue with regard to fiber size and oxidative capacity (9, 10). It was further realized that permanent high-altitude residents (La Paz 3500-4000 m) also have a reduced muscle oxidative capacity and capillarity (6) and that the high oxidative capacity observed by Reynafarje in high-landers was likely due to a difference in training status of the observed populations. The consensus now is that permanent exposition to severe hypoxia has detrimental effects on muscle tissue. It is reasoned that the relative scarcity of oxygen in the atmosphere would lead to a reduction of the structures involved in oxygen utilization (3). We hypothesized that, among other things, compromised protein synthesis could be responsible for the detrimental effect of permanent hypoxia exposition on muscle tissue. As a consequence of these contentions we started to conduct experiments in which hypoxia exposition was limited to the period of the training session while subjects were then left to recover in normoxia (5). The results of this study indicated that training in hypoxia had a similar outcome as training in normoxia with a few notable exceptions. Functionally, we found that training at the same relative workload in hypoxia led to a larger increase of VO_2max when measured in hypoxia; structurally we found an increase in muscle fiber size not observed with endurance training in normoxia. Our findings thus indicated that training in hypoxia could indeed be expected to have specific effects on muscle tissue not seen with normoxia training. Our results were in support of the observations of Terrados et al. (17) indicating not only a larger increase of performance under hypoxic conditions after hypoxia training than after normoxia training – but also a tendency for an increase of capillarity after hypoxia training. The same author (16) later demonstrated in a single leg exercise study that there was a greater increase in citrate synthase activity under hypobaric than under normobaric training conditions as well as an increase in myoglobin concentration in the hypoxia trained leg only.

The physiological implications of altitude training for sea-level performance were extensively reviewed by Bailey and Davies (1). They content that acclimatization to a reduced PiO_2 is a prerequisite to achieve optimal performance in a hypoxic environment. However, they also come to the conclusion that the scientific evidence does not support continuous or

intermittent hypoxic training to be effective in enhancing sea-level performance. On the other hand, their exhaustive review of the literature indicated a noticeable lack of studies with appropriate controls as well as a wide range of exposure altitudes (1640 to 5700m) and exposure times (7 to 63 days).

With the present review we would like to outline the pitfalls in designing altitude training studies. We will concentrate on hypoxia application during training only. This training mode has no effect on the erythropoietin system and thus on blood hemoglobin concentration (5, 7). Training modes capable of elevating plasma erythropoietin levels are dealt with in the accompanying paper of Levine (12). We would like to demonstrate the specifics of the molecular basis of structural and functional changes induced with hypoxic training in muscle tissue of previously untrained male subjects (18). We will further present data from hypoxia training studies involving highly trained athletes with the aim of demonstrating possible functional implications of this particular mode of training for athletic performance at altitude and at sea-level.

TRAINING HIGH – LIVING LOW: CONSIDERATIONS FOR CHOOSING AN EXERCISE PROTOCOL

Training athletes vs. untrained subjects: From an experimenters point of view one would always like to design a study to have a large signal to noise ratio. This would call for using untrained subjects. This approach guarantees a large training response – but at a cost. It is generally recognized that training results achieved with previously untrained subjects are not representative of the situation encountered with athletes (the question at hand). In addition to that exercise in hypoxia poses at least one further problem. Calculating intramyocellular PO_2 from magnetic resonance estimates of deoxymyoglobin Richardson et al. (15) remarked that trained athletes showed lower intracellular PO_2 when exercising in normoxia (3.2 mmHg) than untrained subjects working under similar load conditions in hypoxia (3.8 mmHg). To explain this surprising result it was reasoned that athletes appeared to be more oxygen supply limited whereas untrained subjects depended more on oxygen demand. Assuming that the hypoxia response to exercise training could be a threshold phenomenon (15), the difference in response to systemic hypoxia between athletes and non-athletes could be critical for the outcome of hypoxia training studies (see below). While it seems reasonable to expect studies using untrained subjects to give good insight into the basic mechanisms of hypoxia response, the effectiveness of particular protocols with regard to performance changes in athletes can only be established with athletes.

Exercise intensity and duration: In any study involving hypoxia one is faced with the problem that hypoxia reduces maximal exercise capacity. In order to keep the relative training intensity (i.e. % of maximal heart rate or VO_2max) constant the experimenter therefore needs to reduce the absolute exercise intensity for the group training in hypoxia. This has the undesired side effect of reducing the metabolic turnover (a potential signal) in the hypoxia group. One strategy to control for this aspect asks for four experimental groups. Two high-intensity groups that exercise at the same *relative* exercise intensity in normoxia and hypoxia, and two low intensity normoxia and hypoxia training groups. Under these conditions it is possible to arrange the work loads such that two groups work at the same *absolute* exercise intensity, i.e. one in hypoxia and one in normoxia (see Fig. 1). Additionally, one would like to have a control group that is exposed to hypoxia but does not train and possibly one group that is also exposed to hypoxia but trains under normoxia. To establish the effect of hypoxia *per se* on skeletal muscle tissue seems to be worthwhile since hypoxia alone is capable of doubling vascular endothelial growth factor (VEGF) mRNA in muscle of resting rats (2). For obvious logistic (and ethical) reasons there have been no studies to our knowledge that exploited all of these permutations of hypoxia exposure and training intensities in humans.

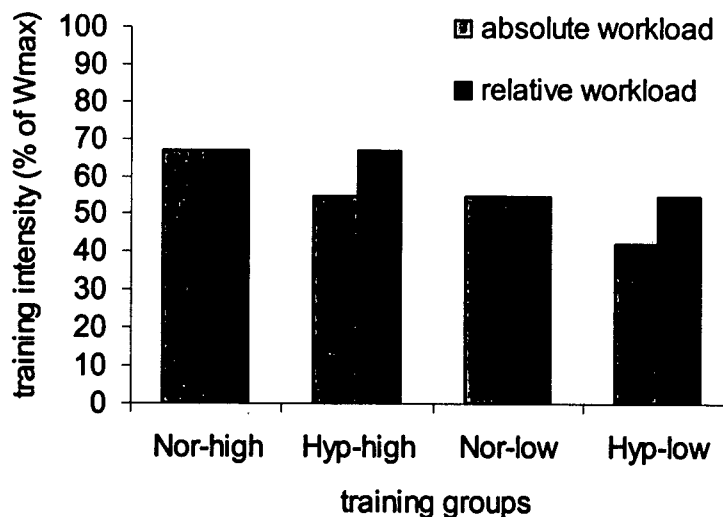


Figure 1. Design of a study with four groups, two trained at a high-intensity level, one under normoxic (Nor-high) and the other under normobaric hypoxic conditions (Hyp-high). The other two groups trained at low-intensity, one under normoxic (Nor-low) and the other in normobaric hypoxic conditions (Hyp-low). Hyp-high and Nor-low groups trained at the same percentage of normoxic maximal power output (e.g., similar absolute values of ATP-turnover). High-intensity training groups and low-intensity training groups trained at the same percentage of maximal power output in each condition (e.g., similar relative values of ATP-turnover).

Training studies of previously untrained subjects typically last for 6 weeks to 2 months. This is generally too long and too much of an intervention for a study involving internationally competitive athletes. In studies with trained subjects the hypoxia intervention is therefore in most cases shorter (4, 4 weeks; 17, 3-4 weeks; 13, 10 days). With highly trained athletes it is also necessary to consider how much of the total training time should be spent under hypoxic conditions. Under the assumption that competitive endurance athletes are always under the risk of overtraining and that hypoxia is a significant additional stressor, this question needs to be addressed carefully. In some studies, incidentally those with a positive outcome (13, 17) the athletes performed no additional training to the 60 to 120 min of hypoxia work per day.

Training altitude: Again, the experimenter would like to have a large signal to noise ratio and therefore has a tendency to choose extreme conditions. On the one hand his desire is constrained by the ethical committees, on the other hand by the compliance of subjects. In particular, trained athletes seem to suffer more from hypoxia than untrained controls both objectively (8) and subjectively (our experience). Training altitudes in "successful" hypoxia training studies with athletes have been rather low (17, 2300m; 13, 2500m). This moderate degree of hypoxia may be sufficient to elicit a hypoxia response in athletes possessing a large peripheral oxidative capacity and thus a low intramycellular PO_2 as indicated above (15). In our practical experience it seems necessary to consider additionally the normal living altitude of athletes. In Switzerland a number of our top skiers (alpine or cross-country) live at altitudes between 1000 and 1600m. Although we have no hard data it would seem obvious that athletes that train and live at these altitudes for a good part of the season need an additional stimulus. In our studies with athletes we have therefore pragmatically chosen a training altitude of 3200m.

A final consideration when subjecting athletes to hypoxia training is the individual hypoxia response. Testing elite cross-country skiers at different altitudes we found a large variability of loss of performance capacity (maximal power, W_{max} ; maximal oxygen consumption, VO_{2max}) ranging from 3 to 15% at 1800m testing altitude (Fig. 2). It has further been shown with the training paradigm "living high-training low" (4) that there is a wide variability of the individual response. Chapman et al. (4) distinguished a group of responders in which the erythropoietin (Epo) response 30 hours after exposition to hypoxia was significantly larger than in non-responders. After 14 days at altitude Epo was still elevated in responders but had returned to baseline values in non-responders. Incidentally, non-responders trained at lower intensities at altitude and did not increase VO_{2max} or red cell volume significantly. A possible explanation of this finding could reside in a polymorphism of genes responsible for the "hypoxia response". No such

polymorphism has been described yet, however, our own experience also points toward a large interindividual variability of the response to a hypoxia training challenge. In the absence of any modality to test for this we are carefully monitoring the individual subjective and objective response to exercise in hypoxia. We have currently not enough data to decide whether the athletes that loose much performance capacity when measured in acute hypoxia are those who respond well to an altitude training or vice versa.

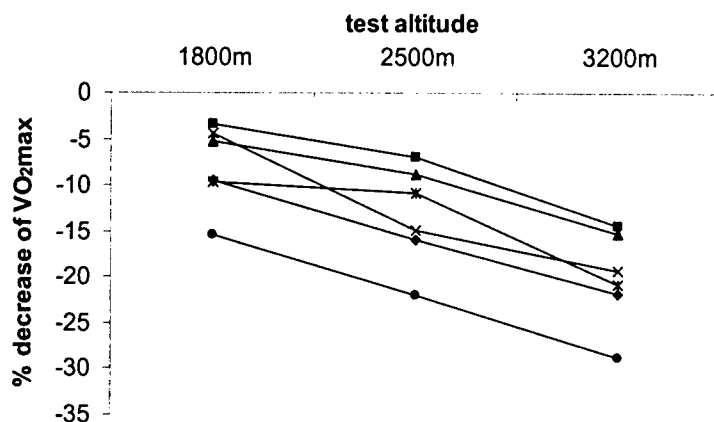


Figure 2. Percent decrease of VO₂max during acute altitude exposition in elite cross-country skiers compared to values at 500m. The graph demonstrates the large variability in loss of aerobic performance capacity.

TRAINING IN HYPOXIA; THE RESPONSE OF UNTRAINED SUBJECTS

We have trained 30 physically active, but not systematically endurance trained subjects for 6 weeks, 5 times per week for 30 minutes on calibrated bicycle ergometers in four groups (Fig. 1). Training was performed at high intensity (4-6 mmol/l plasma lactate) in normoxia and hypoxia at the same relative training intensity (66 to 67% of W_{max} in normoxia or hypoxia) or at low intensity (2-3 mmol/l plasma lactate or 58 and 52% of W_{max} in normoxia or hypoxia). The training loads were chosen such that the hypoxia high-intensity and the normoxia low-intensity group worked at similar absolute workloads (54 and 59% of normoxic W_{max}). The functional results of this study as well as the molecular and structural changes of skeletal muscle tissue in these subjects have recently been published (7; 18). We would like here to summarize some key results which give insight into the differences of the systemic and local skeletal muscle tissue response when subjects train under normoxic vs. hypoxic conditions.

Functional results: VO_2max measured in normoxia increased similarly in all groups, independent of altitude or training intensity between 9 and 11%. However, when VO_2max was measured at the training altitude (3850 m) the two normoxia groups only increased VO_2max by some 3% while the hypoxia group showed increases of 7%. Similar results were obtained for maximal power achieved in the VO_2max tests. Both hypoxia groups performed significantly better than the normoxia groups when tested in hypoxia but not when tested in normoxia. Apart from the obvious advantage hypoxia training had for performance at (simulated) altitude, it is also of note that in previously untrained people we found very similar functional improvements independent of the training intensity.

Structural changes: We found a small but significant 5% increase in skeletal muscle volume of the knee extensor muscles (measured with magnetic resonance imaging), in the hypoxia high-intensity group only, confirming previous results of Desplanches et al. (5). Volume density of mitochondria increased in all groups significantly by 11 to 54%. Both training intensity and hypoxia had significant effects on the increase of muscle oxidative capacity with the largest increase in muscle mitochondrial volume recorded for the hypoxia high-intensity group. Capillarity was assessed by estimating capillary length density (i.e. capillary length per unit volume of skeletal muscle fiber; an unbiased estimate of capillary length). We found capillary length to be increased in the high-intensity hypoxia group only.

Molecular changes: The changes in the concentration of mRNA and thus likely the transcriptional activity of a number of selected genes is reported in Fig. 3. Heat shock protein 70 (HSP 70) indicates that muscle tissue experienced a significantly larger stress with high intensity exercise than with low intensity exercise independent of training altitude. By contrast, HIF-1 α was upregulated independently of exercise intensity by exercise in hypoxia only. The constitutively expressed HIF- β (or ARNT) showed no difference in mRNA concentration with any of the interventions. Oxidative enzyme mRNA (nuclear-coded, Cox 4 and SDH; mitochondrial-coded, Cox 1 and NADH6) also showed a consistent pattern of changes. They showed no increase with low-intensity training in normoxia, a significant but somewhat varied increase with low-intensity hypoxia training and the largest increases with high-intensity training, in particular with high-intensity hypoxia training. Of interest were the changes in myoglobin and VEGF mRNAs. Both transcripts were increased in hypoxia high-intensity exercise only, indicating that possibly mechanical (or metabolic) stimuli as well as a hypoxia signal seem to be necessary to elicit the myoglobin and VEGF responses. The capillary structural data are in direct support of the VEGF mRNA data whereas so far we have not obtained myoglobin protein data. In situ hybridization for myoglobin mRNA (unpublished observations)

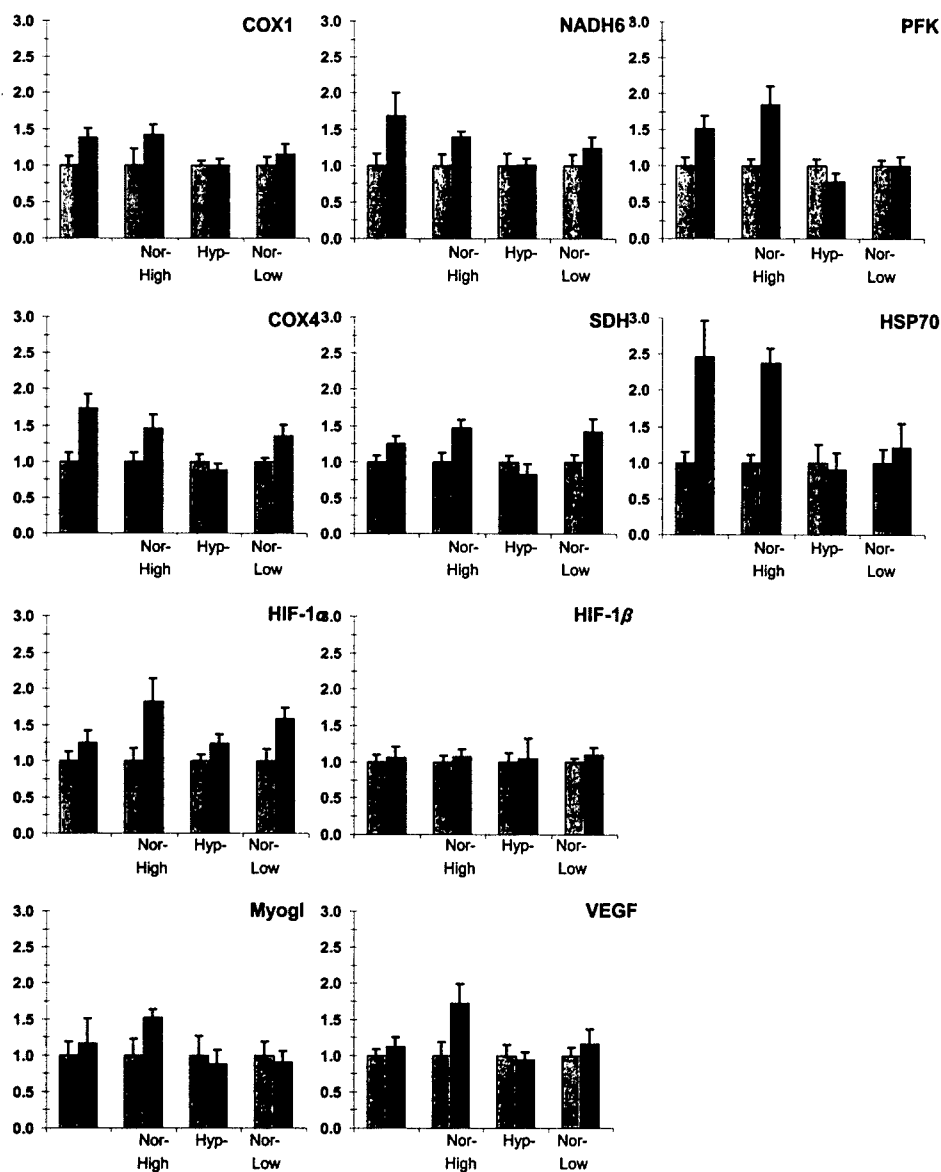


Figure 3. Changes in mRNA concentration in biopsies of vastus lateralis muscle after training for 6 weeks in hypoxia or normoxia at two different exercise intensities (for identification of groups see figure 1). Values after training are presented in relation to pre-training values which are normalized to 1. * $p < 0.05$, significant difference between pre- and post training values; (*) = $p < 0.10$, (means \pm SEM).

indicates that fast twitch fibers, showing a weaker myoglobin mRNA signal before training, may respond preferentially to training in hypoxia. The qualitative data indicate a more homogenous myoglobin mRNA expression across all fibers after training in hypoxia. Interestingly PFK (an established HIF downstream gene) mRNA expression was also increased significantly more in hypoxia high-intensity training than in high-intensity normoxia training.

Summarizing the exercise response of previously untrained subjects we find that functional parameters are relatively insensitive to training conditions and that changes of global variables of exercise capacity with hypoxia training can be demonstrated only when testing is done in hypoxia. Structural variables give a more detailed picture of the training response showing advantages both for training at higher intensities and under hypoxia. Looking at the changes of the transcriptome offers the opportunity to study the training response with unprecedented resolution. Changes in the expression pattern of early genes, structural proteins and metabolic enzymes of any metabolic pathway can be traced with relative ease. For the training response as outlined in this example we can identify a “hypoxia signature” with the HIF- system upregulated exclusively in hypoxia and established downstream genes such as VEGF and PFK upregulated with the combination of hypoxia and exercise. The response of other established downstream genes of HIF needs to be followed up (20).

TRAINING IN HYPOXIA: THE RESPONSE OF TRAINED SUBJECTS

We have carried out a hypoxia training study in 9 male elite alpine skiers of the Swiss National Team (19). 4 athletes performed 8 to 16 hypoxia training sessions over a period of 2 months while their colleagues followed the normal team training schedule. The hypoxia training sessions replaced all endurance work (above 60% of the maximal heart rate) but not other aspects of training such as technical work. Hypoxia training consisted of 30 min of cycling on an ergometer at an altitude of 3200m at an intensity corresponding to the individual anaerobic threshold (heart rate 90% of HR max, plasma lactate 4-6 mmol/l). Performance tests were carried out before and at the end of the intervention period. As indicated in Fig. 4, VO_2max increased when measured at 500m, 1800m, 2500m and 3200m in the hypoxia trained group only. Maximal power output was significantly improved by 5.5%. Evidence for an altitude specificity of hypoxia training is demonstrated in Fig. 5. Lactate concentrations and Borg ratings decreased significantly at the higher exercise intensities in the hypoxia trained group – but only when measured at the training altitude. When measured in

normoxia neither of the groups showed an improvement in these two variables. All of the athletes tolerated the hypoxia protocol well and indicated to have profited from the hypoxia training intervention.

This study has the obvious limitation of a small number of subjects. Moreover, we were unable to have the normoxia group perform an identical number of workouts on the bicycle ergometer in normoxia. It was also impossible to blind subjects with regard to the hypoxia intervention. However, these limitations seem unavoidable when experimenting with competitive athletes and the results must be qualified accordingly. With these limitations in mind we contend that training in hypoxia can be beneficial for athletes when preparing for competition at altitude. In view of the fact that the competition calendar does not in general allow for a classical altitude acclimation period before major competitive events held at altitude (such as the Olympic Games 2002 in Salt Lake City), hypoxia training may indeed confer certain advantages to athletes. Commercially available hardware (i.e. AltiTrainer 200, LMT, Wallisellen, Switzerland) allows for hypoxia training anywhere, under the condition that compressed nitrogen is available.

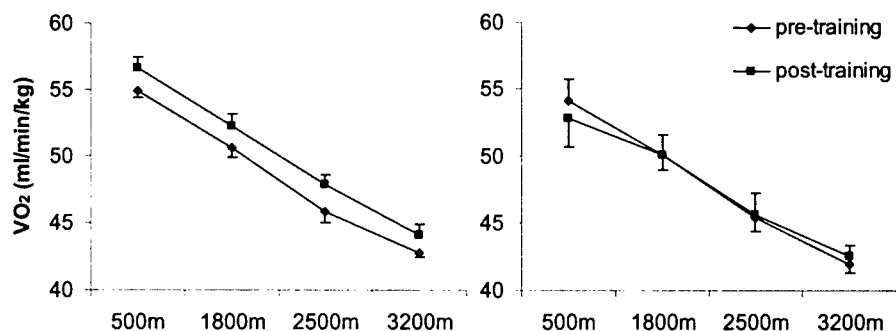


Figure 4. Change of VO_2max at different altitudes with 6 weeks of hypoxia training in four top level alpine skiers. Five skiers served as control performing normal endurance training. Mean values \pm standard error are given.

CONCLUSIONS AND OUTLOOK

“Training high–living low” and “living high–training low” seem to be mutually exclusive, disparate training paradigm. This is not the case. In both cases, the exposure time of athletes to a hypoxic environment is limited to achieve a distinct (but different) physiologic goal. In the case of “living high–training low” this goal is an increase in systemic hemoglobin concentration and thus an improvement of aerobic work capacity. With the concept “training high–living low” it is an improvement of skeletal muscle tissue

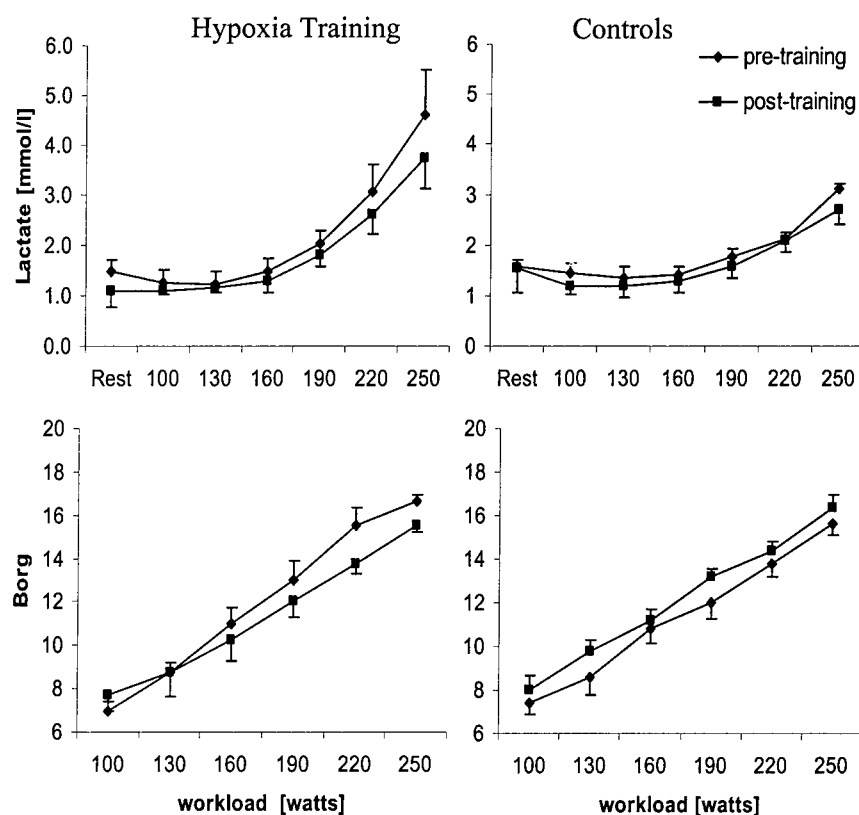


Figure 5. Results of VO_2max test carried out at a simulated altitude of 3200m, indicate an improvement of a) plasma lactate values and b) rating of perceived exertion (Borg scale) in the hypoxia trained group only. VO_2max tests in normoxia (500m) showed no improvement in these variables for neither of the groups. Mean values \pm standard error are given.

with regard to the transfer characteristics for oxygen useful for competitions at altitude but with possible benefits also for sea level performance. A practical goal for the future would be to find ways to combine these two approaches for the greater benefit of athletes. To this end the training conditions in terms of training altitude, time of exposure and training intensity need to be better characterized. On more theoretical grounds, experimenting with exercise in hypoxia will advance our understanding of the molecular mechanisms governing systemic and local responses to challenges of the environment and the way these changes enhance the performance of the organism under specific stress conditions.

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Chapter 7

The effects of altitude training are mediated primarily by acclimatization, rather than by hypoxic exercise

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Abstract: For training at altitude to be effective, it must provide some advantage above and beyond similar training at sea level. This advantage could be provided by: 1) acclimatization to altitude which improves oxygen transport and/or utilization; 2) hypoxic exercise which “intensifies” the training stimulus; or 3) some combination of both. Controlled studies of “typical” altitude training, involving both altitude acclimatization and hypoxic exercise have never been shown to improve sea level performance. This failure has been attributed to reduced training loads at altitude. One approach developed by Levine and Stray-Gundersen, called “living high – training low” has been shown to improve sea level performance over events lasting 8-20 minutes. This strategy combines altitude acclimatization (2,500m) with low altitude training to get the optimal effect. The opposite strategy, “living low – training high” is proposed by Dr. Hoppeler in this debate. In defense of the primacy of the altitude acclimatization effect, data will be presented to support the following: 1). Living high-training low clearly improves performance in athletes of all abilities; 2). The mechanism of this improvement is primarily an increase in erythropoietin leading to increased red cell mass, VO_{2max} , and running performance; 3). Rather than intensifying the training stimulus, training at altitude leads to the opposite effect – reduced speeds, reduced power output, reduced oxygen flux – and, following the principal of symmorphosis, is not likely to provide any advantage for a well trained athlete; 4). At the moderate altitudes used by most athletes, resting oxygen delivery to skeletal muscle is well preserved, arguing against any detrimental effect on “protein synthesis”; 5). It is possible however, that at significantly higher altitudes, acclimatization leads to appetite suppression, inhibition of protein synthesis, muscle wasting, excessive ventilatory work, and metabolic compensation that is NOT advantageous for a competitive athlete.

Key words: erythropoietin, red cell mass, intermittent hypoxia

INTRODUCTION

Altitude training has been used frequently by endurance athletes to enhance performance. However not all athletes or teams have the resources to travel to high altitude environments on a regular basis. Moreover, issues such as availability of adequate training facilities have limited the use of mountain based altitude training. In the last few years, there has been a remarkable increase in the number of techniques designed to “bring the mountain to the athlete.” Nitrogen houses, hypoxia tents, special breathing apparatuses to provide inspired hypoxia during exercise, all have been developed and promoted to simulate the critical elements of altitude training. But what exactly are these critical elements?

For training at altitude to be effective, it must provide some advantage above and beyond similar training at sea level. This advantage could be provided by: 1) acclimatization to altitude which improves oxygen transport and/or utilization; 2) hypoxic exercise which “intensifies” the training stimulus; or 3) some combination of both (24). Controlled studies of “typical” altitude training, involving both altitude acclimatization and hypoxic exercise have never been shown to improve sea level performance. This failure has been attributed to reduced training loads at altitude (36). One approach developed by Levine and Stray-Gundersen, called “living high – training low” has been shown to improve sea level performance over events lasting 8-20 minutes (23, 24). This strategy combines moderate altitude acclimatization (2,500m) with low altitude training to get the optimal effect. The opposite strategy, “living low – training high” is proposed by Dr. Hoppeler in this debate. In defense of the primacy of the altitude acclimatization effect, data will be presented to support the following: 1). Living high-training low clearly improves performance in athletes of all abilities; 2). The mechanism of this improvement is primarily an increase in erythropoietin leading to increased red cell mass, $\text{VO}_{2\text{max}}$, and running performance; 3). Rather than intensifying the training stimulus, training at altitude leads to the opposite effect – reduced speeds, reduced power output, reduced oxygen flux – and, following the principal of symmorphosis, is not likely to provide any advantage for a well trained athlete.

The living high-training low model was confirmed in a series of carefully controlled studies (10, 23, 24, 43) which have a number of important features that deserve emphasis: 1) all studies began with a 2 week “lead-in” phase where athletes were brought from their home cities to Dallas, TX

(150m above sea level) for familiarization with laboratory equipment and testing procedures, and a focused period of controlled training to overcome the training camp effect. This strategy derived from pilot work which showed that training camps generally resulted in an increase in VO_2max and improved performance in collegiate runners regardless of where they lived and trained. Subsequently, in one pilot study designed to determine the minimum duration of training required to observe this effect, 6 male runners increased their VO_2max from 68 ± 2 ml/kg/min to 70 ± 1 ml/kg/min after 2 weeks of supervised training at sea level, but did not increase further after an additional 2 weeks of training (70 ± 2 ml/kg/min) (Levine and Stray-Gundersen, unpublished observations). 2) this lead-in phase was followed by a 4 week mesocycle of training at sea level where all athletes trained together prior to randomization to bring all athletes up to an equivalent degree of training readiness, and to provide a longitudinal control for the experimental intervention. This period also allowed additional time to restore bone marrow iron stores in those athletes who were iron deficient. Previous work by the authors (41, 44) and others (14) demonstrated that individuals who are iron deficient are unable to increase the red cell mass in response to altitude exposure; 3) athletes were then randomized into one of three training groups ($n=13$ for each, 9 men, 4 women) where they were exposed for 4 weeks to: a) the primary experimental group where the athletes lived at 2,500m and traveled down to a lower altitude of 1,250m once or twice per day to train (High-Low); b) an altitude control (High-High), where the athletes lived at 2,500m together with the Hi-Lo athletes, but did all their training at the same altitude or higher (2,500-3,000m); c) a sea level control where the athletes traveled to a new, training camp environment with mountainous terrain, but at sea level altitude (Low-Low). The volume and relative intensity of training was closely matched among groups, and followed the same pattern as the previous 4 weeks of training at sea level. All subjects then returned to sea level for post-intervention testing.

The essential results of these studies are as follows: 1) the groups living at 2,500m had a significant increase in erythropoietin concentration within the first 48 hours of ascent to altitude, which led to a significant increase in the erythrocyte volume (blood volume – plasma volume); neither changed significantly in the sea level control. 2) coincident with the increase in erythrocyte volume, there was an increase in maximal oxygen uptake in both groups living at 2,500m (Figure 1), that was proportional to the increase in erythrocyte volume, and that was not observed in the control group performing similar training in an outstanding training camp environment, but at sea level.

Despite an increase in VO_2 max in both groups of subjects living at moderate altitude, only the group performing all their training at low altitude improved 5,000m racing time by 1.5% (Figure 2).

If both groups of athletes living at 2,500m increased erythrocyte volume and VO₂max, then why didn't both groups improve running performance? Based on previous reports that muscle buffer capacity might increase with altitude exposure (27), one possibility we considered was that "anaerobic"

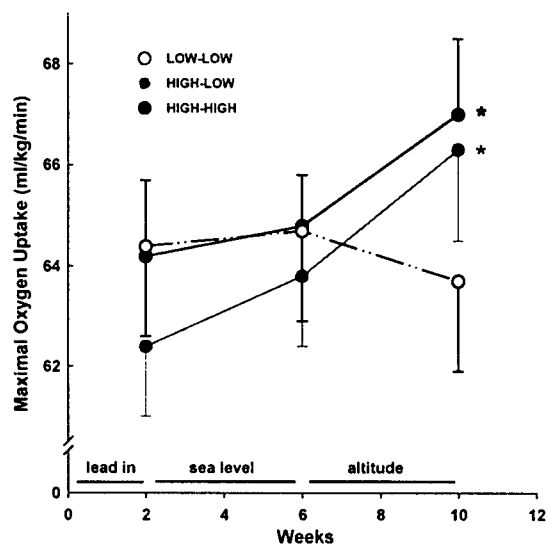


Figure 1. Change in VO₂max after sea level and living high-training low altitude training, with sea level (low-low) and altitude (high-high) controls. From ref (23).

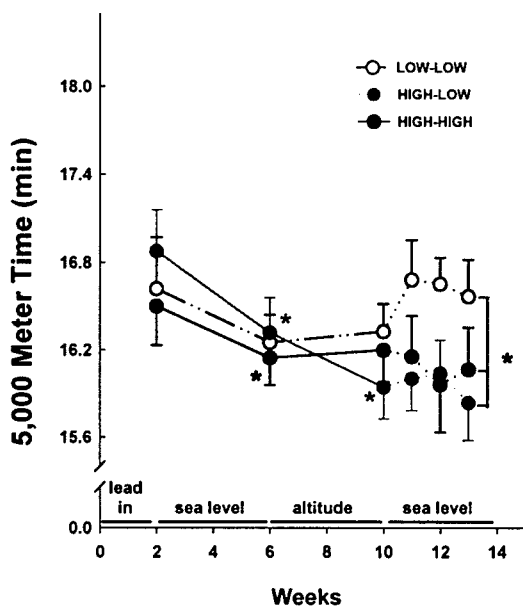


Figure 2. Change in 5,000 m time trial time from ref (23). Note improvement in 5K time in all groups after initial training at sea level, that only improved further in the high-low group. Data shown are for men only because of marked variability in response of some of the control women

performance was improved more by living high-training low. Another possibility raised by some investigators was that running economy might be altered by altitude acclimatization, though data to support this hypothesis are controversial. However in the studies by Levine and Stray-Gundersen, neither the accumulated oxygen deficit (measured by uphill running on a treadmill (26), Figure 3), nor the running economy (measured as the slope of the relationship between running speed at 8, 10, and 12 miles/hr and oxygen uptake) were different among groups, or altered by training either at altitude or at sea level.

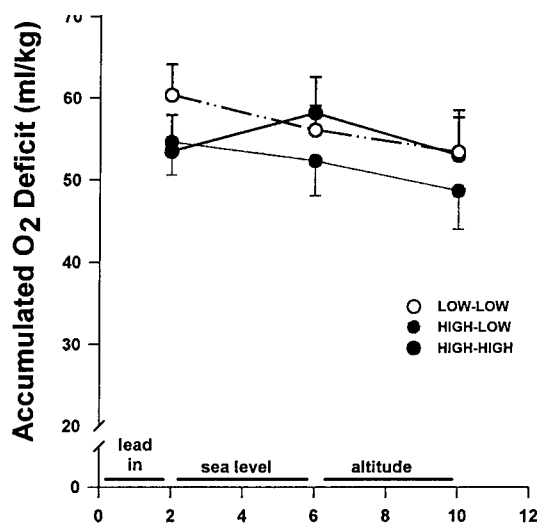


Figure 3. Change in anaerobic capacity, as estimated from the accumulated oxygen deficit during supramaximal uphill treadmill running. Data derived from (23), originally presented only in table form. Symbols are the same as in fig 1 and 2.

The key difference between the high-high and the high-low altitude groups was that the high-low group performed all their training at low altitude, and thus were able to maintain both training velocity, and oxygen flux during high intensity "interval" type training sessions that are essential for the performance of competitive runners. These sessions (1,000m intervals run at 110% of race pace) were run at slower speeds, reduced oxygen uptake, lower heart rate and lower peak lactate in the athletes performing all their training at 2,500m, than the same sessions run either at 1,250m or at sea level (23), as has been noted by the authors in other groups of athletes (24), as well as other investigators (8). For the high-low athletes, this quality of training maintained muscle buffer capacity, which decreased in the athletes attempting to do all their training at moderate altitude (42). Functionally, the preservation of muscle structure allowed an increase in both the VO₂ at the ventilatory threshold, and the velocity at VO₂max which were present only in the high-low group (23).

The essential nature of maintaining speed and oxygen flux primarily during interval training was confirmed in a subsequent follow-up study,

where another group of 13 athletes lived at 2,500m, performed all their base and recovery training at moderate altitude (2,000-3,000m) but performed all their high intensity training at low altitude (1,250m) ("high-high-low"). These athletes had virtually identical improvements in performance compared with the high-low athletes who did all their training at low altitude (Figure 4).

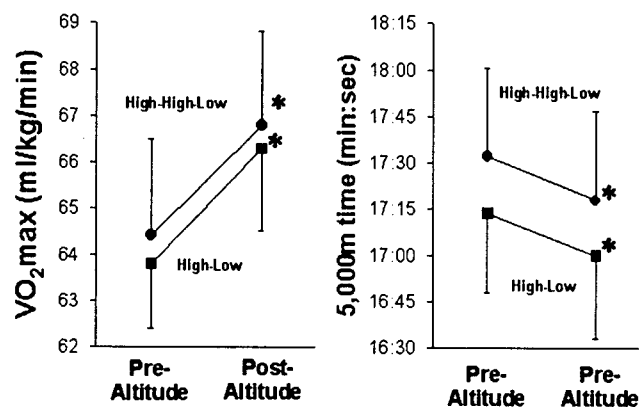


Figure 4. Comparison of 5,000m time trial performance, and VO_{2max} between high-low (all training at 1,250m), and high-high-low (only interval training at 1,250m). High-low data from (23), high-high-low data from (43). Pre-altitude data were obtained after completion of lead in and sea level training phases; $n=13$, 9 men, 4 women for both groups using identical methods.

Not only does this living high-training low strategy work for good collegiate athletes, recent data suggest that it also works for elite athletes. Stray-Gundersen et al. examined 22 elite male and female US distance runners immediately after their national championship competition when they were at their peak performance for the year. After baseline measurements, all performed 4 weeks of living at 2,500m, easy training at 2,000-3,000m and high intensity training at 1,250m (40). Even for athletes who began with VO_{2max} levels above 80 ml/kg/min, the improvement in VO_{2max} and racing performance was similar to that observed in the collegiate athletes, and equivalent between men and women (40).

Despite the clear superiority of living high-training low over traditional altitude or sea level training, there remains substantial individual variability in the magnitude of improvement achieved with such a regimen. At least some of this variability in previous studies, and in the practice of athletes is likely due to iron deficiency. In our experience approximately 40% of competitive distance runners (20% male, 60% in female runners) have a serum ferritin that is suggestive of reduced bone marrow iron stores (41, 44).

When such athletes attempt altitude training, they often do not thrive, and clearly do not increase erythrocyte volume or $\text{VO}_{2\text{max}}$ (22). However even in studies where iron stores were replenished, there remains substantial variability in the outcome of a 4 week altitude training camp. To address the mechanisms of this variability, Chapman et al. (10) performed a retrospective review of all 39 athletes in the Levine and Stray-Gundersen studies (23, 43) who lived at 2,500m and trained between 1,250m and 3,000m, and divided them into two groups: those athletes who improved their 5,000 race by more than the group mean ("responders"); and those that got worse ("non-responders") (Figure 5).

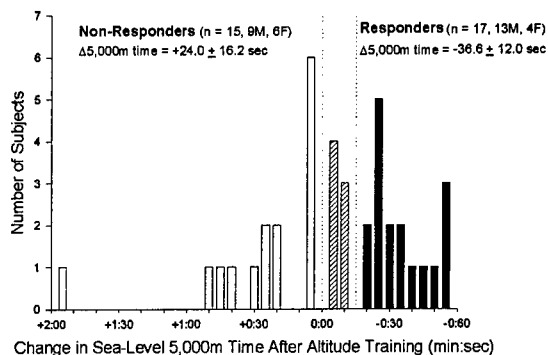


Figure 5. Histogram showing the change in 5,000m run time after 4 weeks of living at 2,500m and training at either 2,500-3,000m, all training at 1,250m, or only interval training at 1,250m (from (10). Hatched bars represent athletes with an intermediate response and were not included in the analysis.

There were no differences between these groups with respect to baseline demographic variables (age, $\text{VO}_{2\text{max}}$, running performance, hemoglobin concentration) or many physiological variables that might determine the magnitude of the acclimatization response to altitude including pulmonary diffusing capacity, oxygen saturation either at rest, during sleep, or during exercise at 2,500m.

However there were a number of key distinguishing features between these two groups. First, although both groups increased erythropoietin concentration after 24 hours at 2,500m, the responders had a significantly greater increase; moreover, the erythropoietin concentration remained elevated after 2 weeks at moderate altitude in the responders (equivalent to the peak response in the non-responders), while it had returned to baseline in the non-responders (Figure 6).

Not only was the increase in erythropoietin more robust in the responders, but this difference appeared to carry substantial physiological significance. Specifically, the responders had an increase in erythrocyte volume, while the non-responders did not. Moreover, this increase in red cells increased aerobic power: the responders had an increase in $\text{VO}_{2\text{max}}$, while the non-responders did not. Finally, the increase in $\text{VO}_{2\text{max}}$ was exactly what would

be predicted from published models quantifying the effect of a change in blood volume and hemoglobin concentration on aerobic power (46): (predicted increase 248 ml/min – actual increase 245 ml/min) (10). Thus the magnitude of the altitude effect is exactly what would be expected from the well known effect of blood doping (9, 11, 48) or exogenous erythropoietin injection (7, 11).

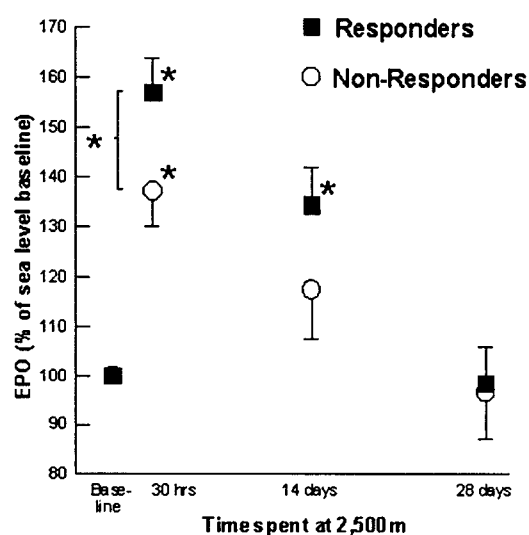


Figure 6. Change in erythropoietin concentration after 24 hours at 2,500m in responders and non-responders, from Chapman et al (10).

In addition to this different erythropoietic response, the responders, regardless of what altitude they trained at, had a smaller decrease in running speed and oxygen uptake during interval training sessions compared to the non-responders. In other words, the responders were better able to maintain normal training velocities and oxygen flux at altitude than the non-responders. These two parallel pathways – the erythropoietic mechanism, and the training quality pathway are pictured in Figure 7, from Chapman et al. (10). Finally, this retrospectively derived formula of distinguishing between responders and non-responders by examining the erythropoietic response to altitude was applied prospectively to an entirely different population of elite athletes, with essentially the same result -- both erythropoietin concentration and VO₂max increased significantly in those “responders” who improved by more than the mean response for the group, while neither increased significantly in those who got slower.

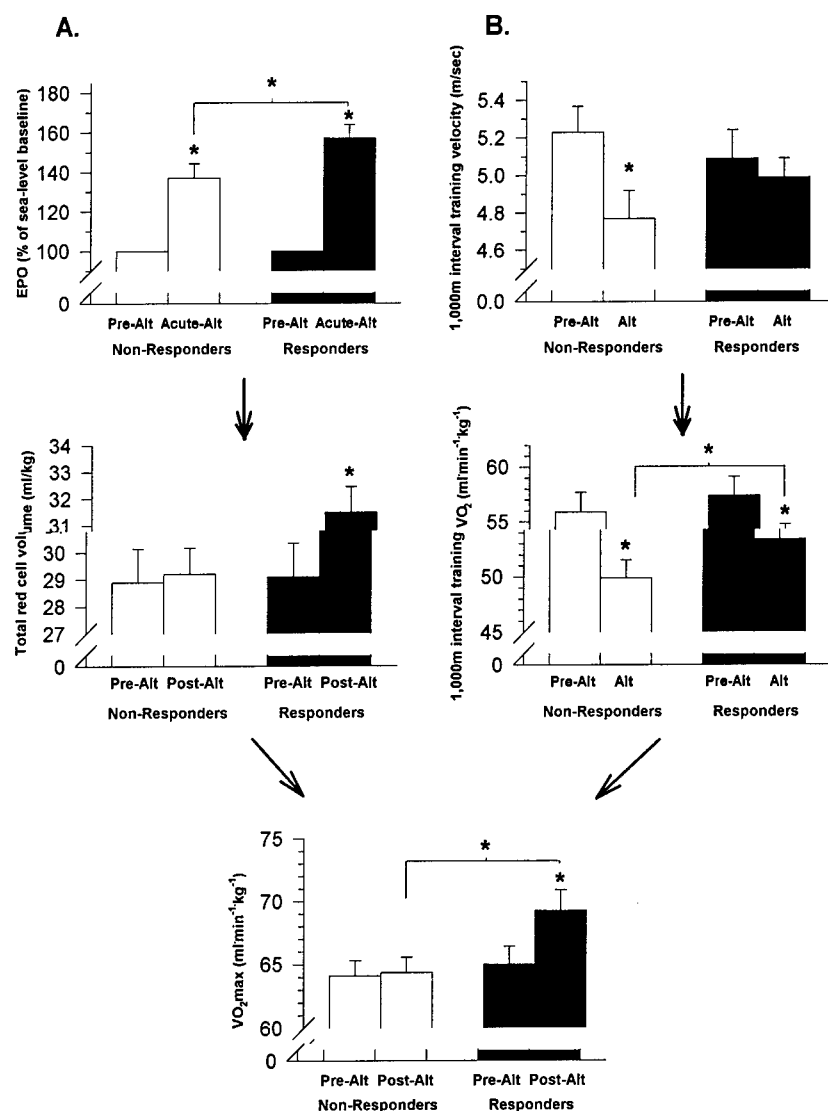


Figure 7. Differences between responders and non-responders with respect to an "altitude acclimatization" pathway involving an increase in epo, increase in red cell mass, and increase in VO₂max (left) in conjunction with a "training quality" pathway involving differences in running speed and oxygen uptake during interval training. Figure from Chapman et al (10).

In summary, these studies demonstrate quite convincingly that: 1) Living high-training low works to improve sea level performance; 2) the mechanism is highly likely to be a stimulation of erythropoiesis leading to an increase in hemoglobin concentration, total blood volume, and aerobic

power; 3) the effect of this increase in oxygen transport capacity is maximized by maintaining normal, sea level oxygen flux during intense exercise avoiding the down regulation of skeletal muscle structure and function that may occur in athletes who attempt to perform all their training under hypoxic conditions.

Some investigators, failing to observe an increase in hemoglobin/myoglobin mass after brief periods of time in normobaric hypoxic environments (8-10 hours/night for 10 days – 3 weeks) have questioned the erythropoietic effect of moderate altitude exposure (4-6). However the evidence in favor of this response is quite compelling. First of all, cross sectional studies in the Peruvian Andes (17, 31, 37), as well as in the Colorado Rockies (47) have demonstrated clearly that there is an elevated red cell mass in natives of high altitude. These studies were done using many different techniques for estimating the red cell mass including radioactive chromium, iron, and phosphorus compounds, as well as Evans Blue and Vital Red dyes, and all show the same result – that is an increase in red cell mass with chronic hypobaric hypoxia. Moreover, by looking at populations living at different altitudes, a graded response has been identified, with an increase in red cell mass that is proportional to the oxyhemoglobin saturation (17, 47).

As expected from the cross-sectional studies, when sea level natives ascend acutely to altitude, there is an increase in iron turnover by more than two-fold, that begins within the first few hours of exposure, and peaks by approximately 2-3 weeks (12, 16, 31). Direct examination of the bone marrow during acute high altitude exposure has documented a dramatic increase in nucleated red blood cells, virtually doubling by 7 days, indicative of accelerated erythropoiesis (16, 31).

Interestingly, both iron turnover (16, 31) as well as erythropoietin concentrations (10, 13, 18, 23, 34) return to sea level values relatively rapidly with chronic altitude exposure. However the red cell mass continues to increase for up to 8 months of chronic altitude exposure, at least at altitudes above 4,000m ((31), Figure 8). Moreover, despite the apparently normal epo levels and iron turnover, it is important to point out that this level of stimulated erythropoiesis is **elevated for the absolute level of the arterial oxygen content**. Thus when altitude natives, or even altitude sojourners return to sea level, there is a suppression of erythropoietin (10, 12, 13, 18, 23, 34), a dramatic reduction in iron turnover and bone marrow production of erythroid cell lines (16, 31), and a marked decrease in red cell survival time (31). This increase in red cell destruction with suppression of epo levels has been termed “neocytolysis” and has been observed under other conditions of a relative increase in oxygen content (1-3, 32, 33).

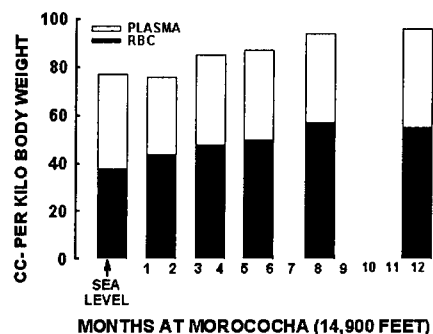


Figure 8. Mean changes in plasma and red cell compartments (measured using Evans Blue dye method) in a group of 10 sea level residents followed over a year of living at 4,500m, from ref 31.

That an increase in oxygen carrying capacity of the blood would be the key altitude mediated adaptation in endurance athletes that might lead to improved sea level performance should not be surprising. The concept of symmorphosis, as elaborated by and Hoppeler and Weibel (15), argues that for any system, such as the respiratory chain for oxygen transport, the maximal capacity of each parameter is adjusted quantitatively to match the structural and functional limits of the demands placed on the system as a whole. Thus, for the "elite athletes" of the animal kingdom, each step of the pathway of oxygen from the atmosphere to the mitochondria has evolved toward optimal function and maximal aerobic power. However humans are somewhat different from more athletic animal species. First of all, unlike horses or greyhounds, humans have a mass-specific mitochondrial oxidative capacity that is greatly in excess of systemic oxygen transport capacity (15). Although at least some of this difference is related to bipedal locomotion, small increases in mitochondrial structure and function are less likely to lead to increases in maximal oxygen transport than increases in oxygen availability. Moreover, "high endurance" animal species have the ability to autotransfuse by splenic contraction and thereby increase the circulating hemoglobin/red cell mass (20), whereas elite human athletes may not. Finally, according to the principles of symmorphosis, the reduced oxygen flux associated with training under hypoxic conditions would be more likely to lead to down regulation of muscle structure and function associated with reduced oxygen transport, rather than up regulation as is hypothesized by proponents of hypoxic exercise as the key component of altitude training.

Thus the evidence is very strong that the primary altitude mediated effect of "altitude training" is the erythropoietic effect of chronic exposure to hypoxia. But how long does an athlete have to live at altitude, or remain in a simulated hypoxic environment to attain this effect. This is one of the key questions that must be answered in the modern era of application of the living high-training low model. The failure of short duration exposures of less than 10 hours for less than 3 weeks to raise red cell mass (4-6) suggests that there is a definite threshold effect, but how this minimal "dose" is

related to the absolute magnitude of hypoxia achieved, duration of exposure/day, or total exposure over time is uncertain. Finnish investigators have been able to demonstrate increases in red cell mass (using the same technique, carbon monoxide rebreathing, as the Australian investigators using shorter term exposures) with 16 hours of hypoxia/night for 4 weeks (21, 35). It is important to recognize that the biological pathways involved in erythropoiesis are complex and non-linear (18). For example, hypoxia stimulates a putative oxygen sensor, which stimulates the production of the hypoxia inducible factor 1 alpha (38, 39, 45), stabilized by the Von Hippel-Lindau factor in the cytoplasm of the renal cortex (25, 30). This complex must be translocated to the nucleus where it initiates the transcription of the erythropoietin gene on chromosome Erythropoietin itself must then circulate to the bone marrow where it binds to the epo receptor which ultimately leads to the acceleration of erythropoiesis (29). Genetic variability clearly plays an important role in both animal (28) and human studies (19) in determining at least some of the variability in the response to hypoxia, and it seems simplistic to expect a simple linear relationship among any of these variables to be easily identified in humans. More work must be done to define the dose-response relationship under these circumstances, and to determine the genetic mechanisms for individual variability.

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Chapter 8

Update: High altitude pulmonary edema

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Abstract: Recent high altitude studies with pulmonary artery (PA) catheterization and broncho-alveolar lavage (BAL) in early high altitude pulmonary edema (HAPE) have increased our understanding of the pathogenetic sequence in HAPE. High preceding PA and pulmonary capillary pressures lead to a non-inflammatory leak of the alveolar-capillary barrier with egress of red cells, plasma proteins and fluid into the alveolar space. The mechanisms accounting for an increased capillary pressure remain speculative. The concept that hypoxic pulmonary vasoconstriction (HPV) is uneven so that regions with less vasoconstriction are over-perfused and become edematous remains compelling but unproved. Also uncertain is the role and extent of pulmonary venoconstriction. With disruption of the normal alveolar-capillary barrier, some individuals may later develop a secondary inflammatory reaction. A high incidence of preceding or concurrent respiratory infection in children with HAPE has been used to support a causative role of inflammation in HAPE. However, alternatively even mild HPV may simply lower the threshold at which inflammation-mediated increases in alveolar capillary permeability cause significant fluid flux into the lung. Other major questions to be addressed in future research are: 1.) What is the mechanism of exaggerated hypoxic pulmonary vasoconstriction? Is there a link to primary pulmonary hypertension? Several observations suggest that susceptibility to HAPE is associated with endothelial dysfunction in pulmonary vessels. This has not yet been studied adequately. 2.) What is the nature of the leak? Is there structural damage, i. e. stress failure, or does stretch cause opening of pores? 3.) What is the pathophysiologic significance of a decreased sodium and water clearance across alveolar epithelial cells in hypoxia? 4.) What is the role of exercise? Do HAPE-susceptible individuals develop pulmonary edema when exposed to hypoxia without exercise? Answers to these questions will increase our

understanding of the pathophysiology of HAPE and also better focus research on the genetic basis of susceptibility to HAPE.

Key words: goals for research, pathophysiology, inflammation, hydrostatic edema, capillary pressure

INTRODUCTION

This paper reports advances made over the last two years regarding our understanding of the pathophysiology of high altitude pulmonary edema (HAPE). The pathophysiological concept at that time is shown in Figure 1 and indicates three major unresolved questions:

What is the role of inflammation?

Analysis of bronchoalveolar lavage (BAL) fluid of mountaineers with HAPE on Mount McKinley (46) and in hospitalized patients with HAPE (31,32) showed in many but not all cases high concentrations of proteins, cytokines, leukotriene B₄ and increased granulocytes. Furthermore, urinary leukotriene E₄ excretion was increased in patients with HAPE reporting to clinics in the Rocky Mountains (29). Susceptibility to HAPE was found to be associated with HLA-DR6 in Japan (23) and with preceding or ongoing upper respiratory tract infection in children in the Rocky Mountains (12). On the other hand, all prospective studies performed in the Alps have revealed no evidence for an inflammatory reaction prior to or during early HAPE.

In-vivo thrombin and fibrin formation (5), transcapillary escape rate of albumin and plasma levels of various cytokines (except for IL6 in beginning HAPE) (30), urinary LTE₄ excretion (4) and exhaled nitric oxide (10) were not increased. The apparent discrepancy may be resolved by the assumption that the inflammatory response in HAPE is secondary to the development of pulmonary edema, since most studies reporting evidence for inflammation were performed in patients who had been in pulmonary edema for one to several days.

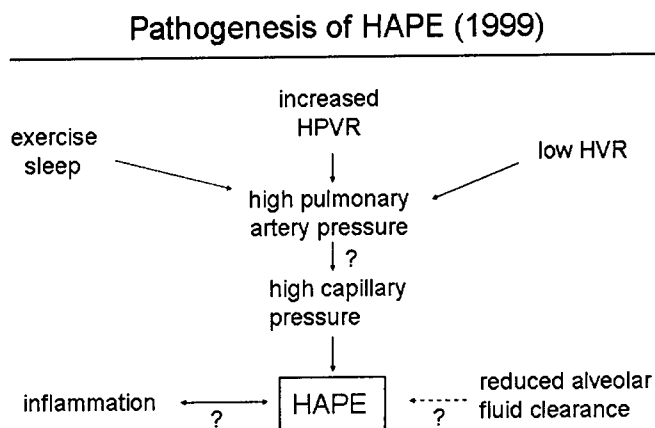


Figure 1. Pathophysiological concept of HAPE, indicating by question marks 3 major questions addressed in recent research.

Which mechanisms account for increased capillary pressure?

It is well established that an excessive rise in pulmonary artery pressure is crucial for the development of HAPE since this pressure rise precedes HAPE (7) and drugs lowering pulmonary artery pressure improve gas exchange in HAPE (19,45) and are effective for treatment (36) and prevention (7) of HAPE. Furthermore, individuals susceptible to HAPE show an exaggerated pulmonary artery pressure rise with hypoxia (27) and exercise (13,18). M. Maggiorini and R. Naeije recently demonstrated that the abnormal rise of pulmonary artery pressure in individuals susceptible to HAPE is accompanied by an increased capillary pressure above 20 mm Hg in those who develop HAPE (34) (Figure 2). This threshold value is in keeping with previous experimental observations in dogs of a PO_2 -independent critical capillary pressure of 17 to 24 mmHg, above which the lungs continuously gain weight (9,26). These results suggest that pulmonary capillary hypertension plays an important role in the pathophysiology of HAPE. In this update, we report additional data that may give clues about the mechanism accounting for the increased capillary pressure.

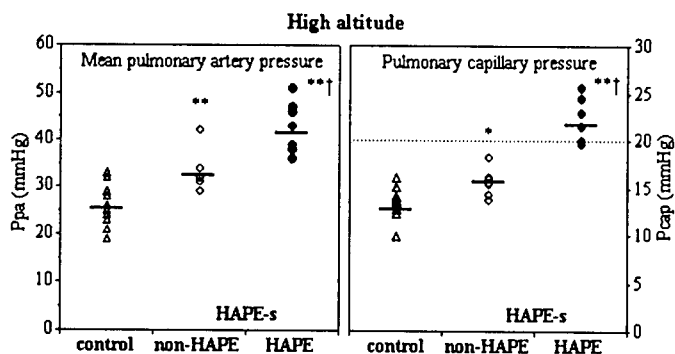


Figure 2. Mean pulmonary artery pressure (Ppa) and pulmonary capillary pressure (Pcap) in 14 controls and in 16 high altitude pulmonary edema-susceptible (HAPE-s) subjects at high altitude. HAPE-s subjects who developed HAPE (HAPE) had all a pulmonary capillary pressure > 19 mmHg in contrast HAPE-s without pulmonary edema (non-HAPE) and controls, whose pulmonary capillary pressures were < 19 mmHg. Bars indicate the mean values in each group. * $p < 0.05$ ** $p < 0.01$ vs. control, † $p < 0.01$ vs non-HAPE.

What is the role of alveolar fluid clearance?

The potential role of alveolar fluid clearance for the pathophysiology of HAPE is covered extensively in chapter 21 in this volume, and thus is not reviewed here.

ROLE OF INFLAMMATION

We performed bronchoalveolar lavage at low altitude (490 m) and at the Capanna Regina Margherita (4559 m) in 8 mountaineers who did not develop HAPE, in 6 mountaineers who developed HAPE 12 – 24 hours after BAL and in 3 mountaineers with radiographically documented HAPE at the time of BAL. The major findings that have been reported in an abstract (48) are shown in Table 1.

Our data demonstrate that HAPE is a hydrostatic pulmonary edema without inflammation at an early stage. Since bleeding accounts for only about 1% of the protein concentration found in alveolar lavage fluid we conclude that overt stress failure (50) with breakage of basement membrane is rare in early HAPE and that pressure-induced openings of pores or fenestrae account for most of the leak.

How do our findings reconcile with data indicating inflammation in HAPE which are reported from the Rocky Mountains, Mount McKinley and Japan? We propose that inflammation is a consequence of HAPE although we have no direct proof for such an evolution in our subjects. However, enhanced thrombin and fibrin formation in advanced cases of HAPE at high altitude (5) and after hospitalization at low altitude (6) are compatible with a secondary inflammatory response. We speculatively present the following concept about the role of capillary pressure and inflammation in HAPE (Figure 3): In susceptible mountaineers with abnormal pulmonary artery pressure response to hypoxia early HAPE is a hydrostatic leak associated with an altered permeability to large molecular weight proteins. This is to be contrasted with the lower pressure leak occurring in acute respiratory distress syndrome (ARDS) which is due to an inflammatory response that is driven by cytokines. It is conceivable, however, that one may develop HAPE with a normal increase of intra-capillary pressure at high altitude when factors enhancing permeability are present, as might be the case during or shortly after an upper respiratory tract infection or in animal models of hypoxic

Table 1. Broncho-alveolar lavage

	550 m		4559 m		
	CONT n = 8	HAPE-S n = 9	CONT n = 8	HAPE-S (well) n = 6	HAPE-S (ill) n = 3
Cell count ($\times 10^6/\text{ml}$)	8.1	6.3	9.5	8.1	9.8
Macrophages (%)	94	95	83	82	85
Neutrophils (%)	1	0	0	0	1
Red cells (% BAL cells)	1	4	6	51*	74*
Total protein (mg/dl)	1	2	14	13	163*
IL-8 (pg/ml)	0.10	0.05	0.18	0.19	0.16
PAP systolic (mmHg)	22	26	37*	61*	81*

* $p < 0.05$ vs. 550 m

Bronchoalveolar lavage (BAL) at 550m and on the second day at 4559m in 8 control subjects (CONT) and in 9 HAPE-susceptible subjects (HAPE-S) of whom 3 had pulmonary edema at the time of BAL. Of those 6 HAPE-S without pulmonary edema at the time of BAL, 4 developed HAPE within 18 hours after BAL.

pulmonary edema using endotoxin priming. Thus, sporadic HAPE may occur in non-susceptible individuals that have a transiently increased permeability of pulmonary capillaries at the time of high altitude exposure.

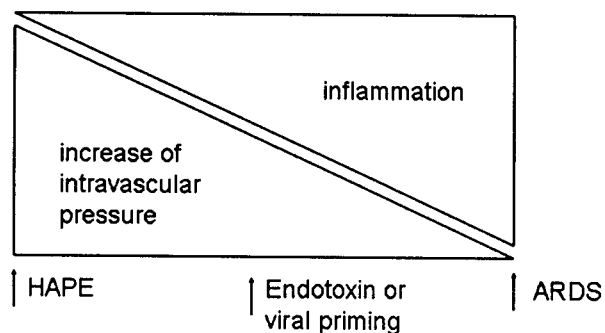


Figure 3. Role of capillary pressure and inflammatory response in various forms of pulmonary leak. For further explanation see text.

CAPILLARY PRESSURE

Mechanisms of fluid filtration

There are two explanations for the occurrence of pulmonary capillary hypertension in high-altitude pulmonary edema-susceptible subjects. The first is inhomogeneous hypoxic vasoconstriction causing regional over-perfusion of capillaries in areas of least arterial vasoconstriction that lead to a protein- and red blood cell-rich pulmonary edema (28), and the second is hypoxic constriction occurring either at the smallest, leaky arterioles or at the venules, or both (19,52). In the isolated perfused pig lung hypoxia causes a quasi parallel upward shift of the Ppa/pulmonary blood flow (Q_p) relationship as well as the relationship between Q_p and filtration rate, suggesting that hypoxia adds a fixed amount of filtration at every increment of Q_p (35). These findings could be explained by an increase in vascular resistance downstream of the region where fluid filtration occurs, most likely in small permeable arterioles and veins. Using the arterial and venous occlusion techniques it has been shown that in pig lungs hypoxia causes a large increase in pulmonary vascular pressure gradient across the "middle" segment (Figure 4) (41). On the basis of comparisons with direct measurements of pressures in 0.9 mm pulmonary arteries and veins and the micropuncture of subpleural arteries and veins (ϕ 30–50 μ m) in the same preparation, it has been shown that the "middle" segment of the pulmonary vascular bed includes arteries and veins that are < 900 μ m diameter but > 50 μ m (Figure 4) (21). Since, there is evidence that the small arterioles are the site of transvascular leakage in the presence of markedly increased Ppa in hypoxia (52) and that pulmonary veins contract in response to hypoxia

(38,55) increasing the resistance downstream of the region of fluid filtration, inhomogeneous vasoconstriction may not be necessary to explain alveolar flooding in subjects with HAPE. However, we still may need to retain some element of regional heterogeneity of HPV (either at arterial and venous sites or both) to explain the heterogeneity of regional edema, at least as we observe it on chest radiographs or CT scans.

Assuming inhomogeneous vasoconstriction one needs to postulate that the tip of the pulmonary catheter in our recent study (Figure 2) always went to pulmonary arteries perfusing edematous lung regions, i. e. low resistance and high flow areas. It is more likely, however, that the constant finding of increased capillary pressure in subjects who develop HAPE is due to the fact that the arterial occlusion method measures pressures in vessels close to 100 μm diameter (21) and that the elevated P_c pressure is the consequence of an excessive hypoxic constriction of pulmonary veins.

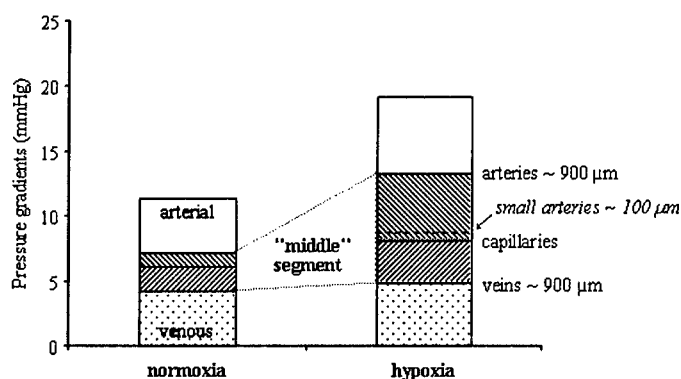


Figure 4. Segmental distribution of pressure gradients in dogs during normoxia and hypoxia as it is obtained using the arterial, venous or both (double) occlusion techniques. Predominately, hypoxia increases the pulmonary artery pressure across the "middle" segment, which includes arteries and veins of $< 900 \mu\text{m}$ but $> 50 \mu\text{m}$. The figure summarize the results published by Hakim et al. (20,21).

Hemodynamic response to inhaled nitric oxide and prostacyclin

Studies using non-invasive pulmonary artery pressure measurements have shown that at high altitude inhalation of nitric oxide (NO) decreases systolic pulmonary artery pressure in high altitude pulmonary edema-susceptible subjects to the levels of non-susceptible subjects (45). This observation has led to the speculation that high altitude pulmonary edema-susceptible subjects may have impaired endogenous NO synthesis. This has now been confirmed with the association of enhanced hypoxic pulmonary vasoconstriction with impaired nitric oxide synthesis (8,10). However, the effect of NO inhalation on high altitude-associated pulmonary hypertension has never been investigated prospectively, or compared to the effects of other vasodilators. In our prospective study, we investigated effects of inhaled NO and inhaled iloprost, a synthetic prostaglandin, on high altitude-associated pulmonary hypertension in 14 controls and 15 HAPE-susceptible climbers after rapid ascent to high altitude (4559 m). Three of the 15 HAPE-susceptible subjects had beginning HAPE on the chest-x-ray at the time of right heart catheterization, and 5 developed HAPE during the following 12 to 24 hours. Heart rate, systemic arterial pressure (Psa), Ppa, and pulmonary artery occlusion pressure (Ppao) were continuously monitored, recorded at end-expiration and stored on a PC for analysis. Pc was obtained using a mono-exponential fitting to the pressure decay curve obtained after rapid inflation of the balloon of the pulmonary artery catheter (16). Cardiac output (Q) was assessed using the thermodilution technique. The percent of the capillary-venous component of total PVR (cv-PVR%) was calculated by the formula: $\{(Pc-Ppao)/Q\}/PVR$. NO was inhaled at a concentration of 40 ppm. 20 μ g (2 ml) Iloprost was inhaled over 8-10 min. using the Respigard-IITM device (particles of 2-6 μ m ϕ). Pulmonary hemodynamics were reassessed at baseline and 10 minutes after the beginning of each intervention. The order of hemodynamic interventions, NO first and Iloprost thereafter, was dictated by the long half-life of the latter (6 hours). At baseline, arterial and mixed-venous blood samples were taken for the measurement of endothelin-1 plasma levels (radioimmunoassay).

After ascent, the mean (\pm SEM) Ppa (26 ± 1 vs. 37 ± 2 ; $p < 0.001$) and Pc (13 ± 1 vs. 19 ± 1 ; $p < 0.001$) were higher in HAPE-susceptible subjects than in controls. The cv-PVR% was 33% in HAPE-susceptible and 25% in controls ($p < 0.01$). Cardiac output was not different between the groups. Effects of inhaled NO and iloprost on pulmonary hemodynamics are shown in Table 2. Inhaled NO and iloprost decreased systemic vascular resistance by an average of 10% ($p = 0.08$). In HAPE-susceptible subjects, inhaled iloprost but not NO decreased Pc and cv-PVR%. In contrast, in subjects resistant to the condition both inhaled vasodilators decreased Pc and cv-

PVR% (Figure 5). There was no difference in the endothelin-1 plasma levels between controls and HAPE-susceptible subjects. Subjects resistant to HAPE had a tendency toward a more pronounced endothelin-1 arterial-venous (mixed-venous) difference (0.58 ± 0.12 vs. 0.24 ± 0.10 pg/ml; $p = 0.04$) than HAPE-susceptibles.

Table 2. Effects of inhaled nitric oxide and iloprost on pulmonary hemodynamics at high altitude

		Baseline	iNO (40ppm)	iPG (20 μ g)
Cardiac index (l.min ⁻¹ .m ⁻²)	Control	3.7 \pm 0.2	4.1 \pm 0.2**	3.9 \pm 0.2
	HAPE-s	3.7 \pm 0.1	4.2 \pm 0.2**	4.0 \pm 0.2*
Mean Ppa (mmHg)	control	26 \pm 1	18 \pm 1**	17 \pm 1**
	HAPE-s	37 \pm 2††	23 \pm 1***†	23 \pm 1***†
Pcap (mmHg)	control	13 \pm 1	11 \pm 1**	11 \pm 1**
	HAPE-s	19 \pm 1††	14 \pm 1***†	12 \pm 1**§
PVRI (dyn.sec.cm ⁻⁵)	control	359 \pm 25	161 \pm 14**	153 \pm 11**
	HAPE-s	614 \pm 42††	264 \pm 24***†	283 \pm 48***†

Effects of inhaled nitric oxide (iNO) and iloprost (iPG) on pulmonary hemodynamics in 14 control and 15 high altitude pulmonary edema susceptible subjects (HAPE-s). Ppa, pulmonary artery pressure; Pc, pulmonary capillary pressure; PVRI, pulmonary vascular resistance index. Values are given as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. baseline; † $p < 0.05$, †† $p < 0.01$ vs. control, § $p < 0.05$ iNO.

In conclusion, the present study shows that in HAPE-susceptible subjects, neither inhaled NO nor iloprost decrease mean Ppa to the levels of controls and that iloprost but not NO decreases cv-PVR% to normal levels. Less pronounced endothelin-1 arterial-venous difference in HAPE-susceptible subjects suggest more endothelin-1 receptors in these subjects and therefore an enhanced uptake leading to stronger vasoconstriction.

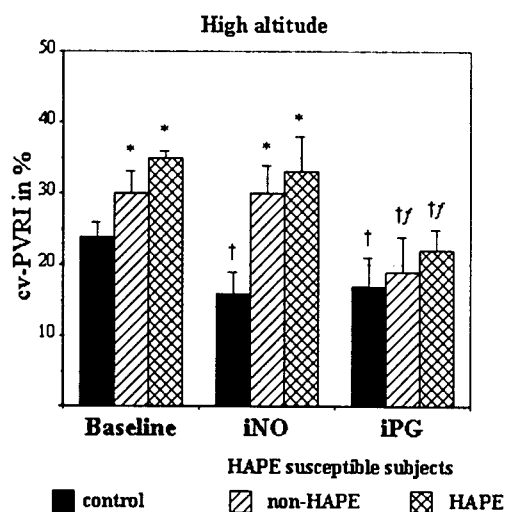
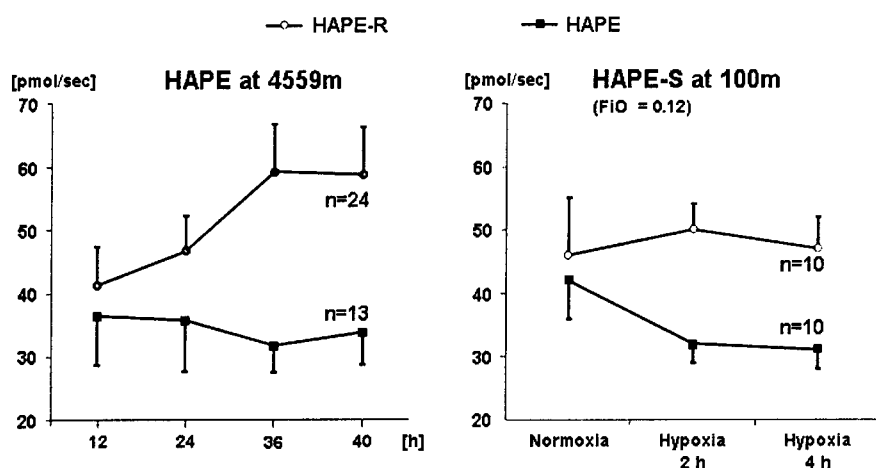


Figure 5. Effects of inhaled nitric oxide (iNO) and iloprost (iPG) on the capillary-venous component of the total pulmonary vascular resistance (cv-PVRI%) given in percent in 14 controls and in 15 high altitude pulmonary edema-susceptible (HAPE-s) subjects at high altitude. Iloprost but not iNO decreased cv-PVRI% in both HAPE-s subjects with (HAPE) and without high altitude pulmonary edema (non-HAPE). Values are given as mean \pm SEM; * p at least < 0.05 vs. control; † vs. baseline; ‡ vs. iNO.

Regulation of pulmonary vascular tone

Lower exhaled NO concentrations in HAPE-susceptible subjects after acute exposure to normobaric hypoxia and at high altitude suggest (Figure 6) impaired NO synthesis in these subjects. However, there is evidence that exhaled NO is not a reliable marker of vascular endothelial function in healthy subjects. We found that inhaled NO did not normalize Ppa in HAPE-susceptible subjects, but did so in those resistant to this condition. In contrast to previous studies using non-invasive measures of systolic pulmonary artery pressure, our findings suggest that impaired NO synthesis alone is not responsible for excessive pulmonary vascular reactivity in HAPE-prone subjects at high altitude. It is likely that additional factors such as sympathetic activity (11) and arachidonic-acid metabolite concentrations in the lung (46) also contribute to increased Ppa in HAPE-susceptible subjects.

Exhaled Nitric Oxide



Duplain et al, AJRCCM 162: 221 – 224, 2000

Busch et al, AJRCCM 163:368-373, 2001

Figure 6. Exhaled NO during 40 hours at 4559 m in individuals developing HAPE and controls (left panel) and in individuals with and without susceptibility to HAPE during 4 hours of exposure to hypoxia (FIO₂ = 0.12) at low altitude.

Animal models have shown that in hypoxia the venous side of pulmonary circulation account for 20 to 25% of the total PVR (1,24). In the present study, the capillary-venous component of total PVR was 25% in controls and 33% HAPE-susceptible subjects, which suggests elevated venous resistance in the latter group. Moreover, inhaled iloprost but not NO decreased cv-PVR% in HAPE-susceptible subjects. Increased vascular resistance in pulmonary veins located more distal may explain this result. The failure of NO to decrease cv-PVR% may be attributed to rapid inactivation due to the high affinity of hemoglobin for NO (22). Comparison of inhaled NO with the non-selective NO donor sodium nitroprusside (SNP) also showed similar results. As assessed by vascular occlusion techniques in blood perfused lungs of rats and 1- to 3-month-old lambs, both SNP and inhaled NO dilated small resistance arteries and veins, whereas SNP only decreased resistance in large pulmonary veins (42,49).

In conclusion, the hemodynamic results obtained in subjects prone to develop HAPE are consistent with increased pulmonary vascular resistance on both the arterial and the venous side, the resistance in the venous segment being probably caused by hypoxic constriction of larger pulmonary veins. Increased sympathetic activity (53), arachidonic-acid metabolites (20) and elevated endothelin-1 plasma levels (2) may also contribute to increasing resistance in the venous segment of the pulmonary circulation. Further

studies are needed to investigate whether in HAPE-susceptible subjects endothelin-1-mediated vasoconstriction is attributed to higher plasma levels (44) or to an enhanced uptake of endothelin-1 by pulmonary circulation, as suggested by the endothelin-1 measurements in our study.

GOALS FOR FUTURE RESEARCH

A pathophysiological model of HAPE has to account for the following clinical observations: a) Variable susceptibility between individuals (3); b) Increasing prevalence with increasing altitude and/or faster rate of ascent (3); c) HAPE occurrence predominantly within the first 5 days at a given altitude and rarely thereafter (25); d) resistance to HAPE within a few days after recovery from an episode of HAPE (33); and e) rapid resolution at low altitude without sequelae (3)

Morphology and mechanism of the leak

In rabbit lungs perfused and fixed under high pressure capillary stress failure (50) has been shown to occur: ruptures of epithelial and endothelial layers of the alveolar wall start to occur at a transmural pressure gradients above 30 cm H₂O and increase with further rise in pressure which will also cause ruptures of basement membrane. Such changes were found in the lungs of some rats exposed within 30 minutes to altitudes of 8800 m (51). Such stress failure could explain alveolar hemorrhage but should lead to an activation of platelets and blood coagulation due to exposure of basement membranes. However, as pointed out already, BAL fluid analysis in early HAPE indicate that hemorrhage only accounts for about 1% of the protein found in BAL fluid and platelet activation was not detectable in early HAPE (5). Thus, it remains questionable whether stress failure is the predominant mechanism of the leak in HAPE, especially of early and mild cases. Experiments in animal models with a time course of exposure and pulmonary artery pressure response that match the typical setting in which HAPE in humans occurs may help to answer this question.

Endothelial dysfunction in the pulmonary circulation

Exhaled NO was found to be decreased in HAPE-susceptible individuals vs. controls during exposure to hypoxia (FIO₂ = 0.12) (8), as well as prior to and during the development of HAPE at high altitude (10) (Figure 6). Furthermore, in the BAL fluid of our study (48), nitrates and nitrites were also decreased in HAPE-susceptible individuals vs. controls, indicating a

decreased NO production in the lungs of the former group. Although exhaled NO is not an accurate indicator of NO produced by endothelial cells (43), these findings, nevertheless, are compatible with decreased NO synthase activity in the lungs of HAPE-susceptible individuals that might also occur in the pulmonary vessels. Increased plasma levels of endothelin in HAPE-susceptible individuals in response to reduced NO, or vice versa, are compatible with the notion of pulmonary endothelial dysfunction in individuals prone to HAPE (44).

Mechanisms accounting for increased pulmonary capillary pressure

The hypothesis of inhomogeneous hypoxic vasoconstriction (28) needs to be tested in humans with imaging techniques of the pulmonary circulation that have high resolution (SPECT, CT or PET), or in animal models by microsphere techniques. Ideally these techniques should be combined in the same subjects with imaging of lung density, as a marker of interstitial and alveolar fluid accumulation. Such co-registration may make it possible to determine whether edema develops in areas of highest flow (least hypoxic vasoconstriction) thus supporting the concept of inhomogeneous hypoxic arterial vasoconstriction and over-perfusion edema, or occurs in areas of least flow (greatest vasoconstriction) endorsing a greater role for pulmonary venoconstriction. Since not all vasodilators work in exactly the same fashion, as shown by the differences between inhaled NO and iloprost (vide supra), careful studies with direct vasodilators and inhibitors of vasoactive substances, such as endothelin, should help to clarify the sites and mechanisms of hypoxic vasoconstriction in humans.

Role of exercise

Exercise leads to an increased pulmonary capillary pressure (13) by two different mechanisms: Increase of pulmonary blood flow may lead to over-perfusion. Further rise of exaggerated pulmonary artery pressure will shift the intraventricular septum towards the left ventricle and impair its filling (39). Therefore, the question arises whether HAPE will occur in susceptible individuals without exercise (i.e. in a hypobaric chamber). This question becomes important for screening families for susceptible members in the context of genetic analyses.

Role of alveolar fluid clearance

β_2 agonists enhance alveolar to blood transepithelial sodium and water transport (14). However, these drugs also lower pulmonary artery pressure (15,47), increase the ventilatory response to hypoxia (54), and tighten cell-to-cell contacts (37), all of which are likely or proven preventive strategies in HAPE. Thus, while using such drugs may well be an optimal therapeutic approach for HAPE, they cannot be used to unequivocally assess the contribution of alveolar fluid clearance in the genesis of HAPE. More selective stimulants of alveolar sodium transport (such as corticosteroids) or inhibitors in animal studies (such as amiloride and its analogs) are necessary to investigate this question.

Genetic basis of HAPE

Research should be directed to gene polymorphisms that account for functional differences in enzyme activities that might play a role in the pathophysiology of HAPE, such as gene polymorphisms that influence the

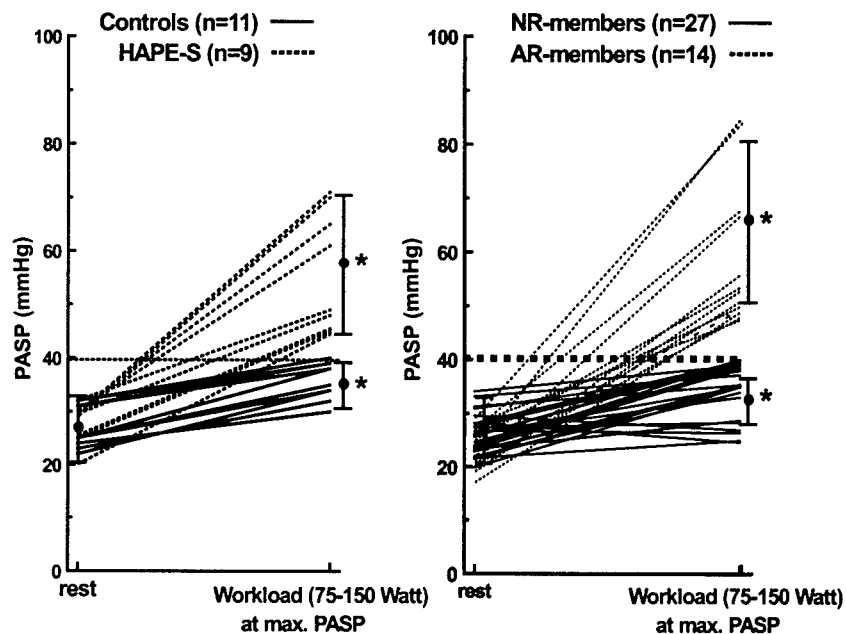


Figure 7. Systolic pulmonary artery pressure measured by doppler-echocardiography before and during exercise in HAPE-susceptible individuals and controls (graph on left side) and in healthy carriers of the PPH-1 gene and in family members without the PPH-1 gene. Data from references 17 and 18.

activity of angiotensin-converting enzyme or nitric oxide synthesis. In addition, the association found between HAPE susceptibility and human leukocyte antigen (HLA) DR6 found in Japanese should also be examined in Caucasians. Furthermore, examinations of families of susceptible individuals ought to address markers of susceptibility like increased hypoxic pulmonary vasoconstriction in order to establish a basis for linkage analysis.

It may be interesting to note that susceptibility to HAPE and susceptibility to primary pulmonary hypertension (PPH) share some similarities. HAPE-susceptible individuals have an abnormal pulmonary artery pressure rise to hypoxia and exercise (18). An abnormally high exercise response of pulmonary artery pressure was also found in healthy carriers of the PPH-1 gene (17) (Figure 7). These investigators have also preliminary data that demonstrate a similar abnormal pulmonary artery pressure rise to two hours of hypoxic exposure ($\text{FIO}_2 = 0.12$) in clinically healthy PPH-gene carriers and in HAPE-susceptible individuals. What does a similar hyper-reactivity of the pulmonary circulation to exercise and hypoxia in healthy PPH gene carriers and HAPE susceptible individuals mean? The PPH-gene induces a defective receptor for a mitogenic hormone of the transforming growth factor family and thereby enhances vascular wall proliferation and thickening. It could be possible that the abnormal pulmonary vasoreactivity of healthy PPH gene carriers may also be linked to the PPH gene. Perhaps, understanding the genetic basis of hyperreactivity in PPH gene carriers to exercise and hypoxia may lead to understanding the genetic abnormalities accounting for susceptibility to HAPE.

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Chapter 9

Phylogenetic comparison and artificial selection

Two approaches in evolutionary physiology

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Abstract: Interspecific comparison has a long and productive history in physiology. Conceptual and statistical advances over the last 15 years have demonstrated several ways in which comparisons can be enhanced by consideration of phylogenetic information, i.e., empirical estimates of the ways in which organisms are related (evolutionary trees). Choice of species to be compared should be informed by phylogenetic information. For example, a comparison of three species that inhabit high altitude with three that live at low altitude would be suspect if each of the two groups were composed of closely-related species (e.g., within single genera). To avoid such "phylogenetic pseudoreplication," one might instead study species from three different genera, each containing one high-altitude and one low-altitude inhabitant. Unfortunately, many studies have not been so carefully designed, sometimes because organisms were not accessible or because the studies incorporated data from the literature. Fortunately, several new statistical methods correct for problems caused by phylogenetic relatedness and descent with modification, the most common being phylogenetically independent contrasts. Another tool that can be used in comparative physiology is selective breeding, which has been practiced for millennia and applied in scientific contexts for over a century. In the last 20 years, ecological and evolutionary physiologists have begun using selection experiments to study processes of genetic adaptation in physiological and behavioral traits. For example, house mice have been maintained in the cold for multiple generations to see what adaptations may occur naturally in response to reduced ambient temperature ("laboratory natural selection"). Our own laboratory has used selective breeding to create four replicate lines of mice that exhibit high levels of voluntary wheel-running behavior, as well as various morphological and physiological characteristics that cause or allow the elevated locomotor activity. Similar experiments could be used to study adaptation to hypoxia.

Key words: adaptation; exercise; genotype-environment interaction; quantitative genetics; voluntary activity

INTRODUCTION

Evolutionary physiology is a subdiscipline that has developed since the late 1970s (12, 21, 29, 30, 38). It grew partly from the realization that many studies in ecological, environmental, and comparative physiology were somewhat naive with respect to the state-of-the-art in modern evolutionary biology. For example, although physiologists have often been interested in adaptation in the genetic/evolutionary sense (i.e., cross-generational genetic changes that occur as a result of natural selection [9]), many of them have made inferences about adaptation based on studies and data that would be considered insufficient for the purpose by evolutionary biologists (e.g., see 17, 25, 33, 34, 35, 37).

Another impetus for the development of evolutionary physiology was the idea that evolutionary studies could in many cases be strengthened by a more rigorous consideration of morphological, physiological, and biochemical mechanisms that account for variation at the organismal level (i.e., how organisms work). For many evolutionary biologists, the organism is left as something of a "black box." Peering inside the box has often been seen as unnecessary for understanding evolutionary (or ecological) phenomena. An expression sometimes heard is: "We shall assume that the organism works!" It is not that evolutionary biologists disdain mechanism; indeed, detailed and highly technically sophisticated studies of genetic mechanisms of evolutionary change are common. Moreover, studies in evolutionary morphology and biomechanics often examine mechanism in considerable detail (53, 59, 65, 70, 87, 90). Nevertheless, it remains true that evolutionary biologists relatively rarely study physiological mechanism (e.g., see 94).

Evolutionary physiologists also recognize that not all organisms are well suited for evolutionary studies, just as not all organisms are well suited for studies of physiological mechanisms (30, 38). For example, a long generation time places great constraints on the kinds of evolutionary studies that can be undertaken (e.g., nobody is going to do a selective breeding experiment with elephants), just as a small body size places constraints on the types of physiological studies that can be done (e.g., because of limits on the amount of blood that can be drawn or on the size of telemeter that can be carried during normal behavior). Hence, many evolutionary physiologists who once worked exclusively on wild animals have subsequently taken to studying laboratory "model organisms" (e.g., A. F. Bennett's studies of bacteria [10]; M. E. Feder's [28, 31] and R. B. Huey's [e.g., 46] studies of *Drosophila*; A. J. Zera's work on crickets [95]; my own laboratory's work on

domesticated house mice [e.g., 19, 26, 44, 47, 51, 56, 57, 75, 83, 84, 85, 86; and see below]).

Although a shift to the use of model systems may not seem radical or like much of a sea change to either evolutionary biologists (witness the routine use of *Drosophila melanogaster*) or physiologists (routine use of rats, mice, and frogs), it can be almost anathema to some comparative and ecological physiologists. In the latter fields, one tradition has been to choose particular species for study because they are of inherent interest, often because they are "extreme" in some way (see 29, 37, 38). Examples would include organisms that live in environments with extreme physical conditions (deserts, polar regions, high altitude, great depth in the ocean), that are unusually small (shrew, hummingbird) or large (elephant, ostrich), that have peculiar behavior (e.g., feeding on blood, Galapagos marine iguanas that dive and feed on seaweed), or that have an unusual body shape (giraffe, snakes in general). A bias often exists against anything that is not seen as a "real organism." Our own laboratory has sometimes encountered this when trying to publish papers on domesticated house mice. Reviewers have, for example, wondered why we did not study a wild rodent, such as *Peromyscus*. Even when the topics are explicitly physiological or evolutionary, rather than ecological, we have encountered such comments as "so what to people who are interested in 'real' organisms?"

In any case, many evolutionary physiologists use species that, while unusual from the perspective of traditional ecological physiology, are routine for other fields. These model organisms can offer many advantages, such as a wealth of background information, the availability of molecular tools that have not yet been developed for wild organisms, and the ability to rear them in the laboratory as well as conduct cross-generational breeding studies (e.g., 54, 71, 82).

In this chapter, I present an overview of two general approaches that are having major impacts on the field of evolutionary physiology: phylogenetic comparison and selective breeding. Although the origins of these approaches are ancient, and they have been applied previously in physiology (38), applications in modern evolutionary physiology can probably be traced, respectively, to Huey and Bennett's (52) studies of the evolution of thermal physiology in Australian scincid lizards, and to Lynch's selective breeding on nest-building behavior in house mice (58, 61). Several other approaches are common in today's evolutionary physiology (30, 38), including formal and informal optimality models (e.g., 3, 22, 91, 92), but they will not be considered here.

PHYLOGENETIC COMPARISONS

Comparisons of species have been a mainstay of comparative physiology since its inception. They have allowed us to catalog the diversity of physiological processes exhibited by living organisms and also to discover general principles of organismal "design." For example, comparisons of animals of different body size have shown that most physiological rate processes vary in a predictable manner with body size, e.g., larger-bodied species generally have lower heart rates, lower rates of respiration, lower metabolic rates on a mass-specific basis, and longer life spans (13, 18, 81).

But the allometric generalities that have emerged from comparing species of different sizes do not apply in a completely general manner. That is, a single allometric equation does not fit all animals. Rather, we often see different relationships when comparing different evolutionary lineages (clades) of animals, such as squamates (lizards and snakes, which represent an evolutionary derivation from lizards) versus mammals versus birds. Sometimes the scaling exponents (i.e., the slope of a line fitted to double-log transformed data) of these relationships differ significantly, but more often we see shifts in the elevations of the lines. For example, squamates have lower standard metabolic rates and also lower maximal rates of oxygen consumption during forced exercise, even when measured at body temperatures (35-40 Celsius) that approximate those of mammals (88, 93). Differences in field metabolic rates, measured by doubly labeled water, are even greater, as they are also affected by variations in body temperatures and activity levels on both a daily and seasonal basis (69).

Lineage-specific allometric relationships that show similar slopes but different elevations (Y-intercepts) are referred to as "grade shifts," in evolutionary biology. Apparent grade shifts in physiological functions have long been known to comparative physiologists. Some relatively recent and widely cited examples include the putatively higher standard metabolic rates of passerine birds as compared with other birds (but see 41, 74), and the lower metabolic rates of marsupial as compared with placental (eutherian) mammals (18, 81, 93).

Knowing of the existence of clear differences among some evolutionary lineages, most modern comparative biologists would consider phylogeny as a possibly important factor when comparing species. Although they often do not think of such distinctions as passerine versus non-passerine birds, or marsupial versus placental mammals, as "phylogeny," these taxonomic categories nonetheless convey something about evolutionary relationships. Passerines are a particular lineage derived from within the avian family tree; marsupials and placentals are generally thought to represent sister lineages (at least if we ignore fossil groups).

1.1 An Example to Illustrate the Importance of Considering Phylogeny

At this point, it is useful to consider a real data set, one which illustrates the perils of ignoring phylogenetic relationships during data analysis. Figure 1A shows the log-log relationship between red blood cell count and

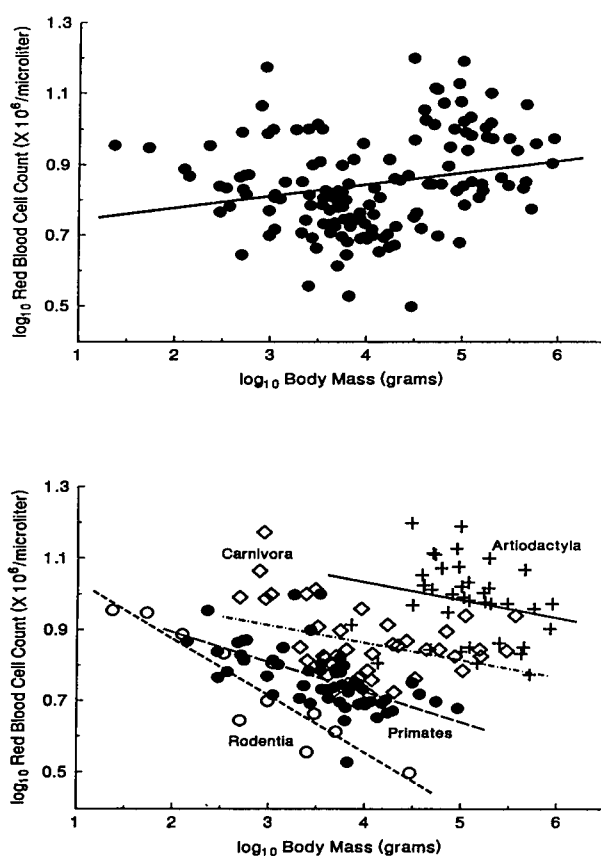


Figure 1. Example of how incorporating phylogenetic information can clarify patterns. Across species of mammals, the number of red blood cells (RBC) per unit volume of blood seems to increase with increasing body size (A). When phylogeny is considered, however (B), negative relationships are apparent within each of four clades, and clades tend to differ in average values. Data from Promislow (72 and pers. comm.).

mass for 146 species of mammals. These data are a subset of those used by Promislow (72). He used a data base compiled from animals living in the London Zoo, so most of the animals had been reared for some period of time under similar environmental conditions. As noted below, this cannot be strictly true for the range of species considered (e.g., mice, monkeys, wolves) because they eat such different diets and have such different housing requirements. Nevertheless, environmental conditions were presumably more similar across species than if each had been captured fresh from the field.

A conventional least-squares linear regression analysis, as is typically used in allometric studies, indicates a statistically significant (2-tailed $P = 0.0057$) positive relationship with a slope of 0.033 (S.E. = 0.012, 95% confidence interval = 0.010 - 0.057). Thus, larger-bodied species tend to have more red blood cells per unit volume of blood. This analysis makes absolutely no reference to the phylogenetic relationships of the 146 species under consideration.

However, as shown in Figure 1B, if we separate the 146 species into their taxonomic orders and perform a conventional analysis of covariance (ANCOVA), we obtain a negative pooled within-groups slope (2-tailed $P < 0.00005$) of -0.077 (S.E. = 0.012, 95% confidence interval = -0.100 - -0.054) and highly significant differences among the orders ($P < 0.00005$). Hence, our conclusions regarding the relationship between body size and the number of red blood cells per unit volume of blood changes when we consider at least a crude representation of phylogenetic relationships, i.e., separating species into their taxonomic orders.

A cautionary note must be added. Many taxonomies do not actually reflect phylogenetic relationships. In some cases, this is because names were assigned before any real knowledge of branching relationships was available. In other cases, names are retained because they have a long historical tradition and appear to convey useful common knowledge. The class Reptilia is a good example. As used traditionally, Reptilia includes living crocodilians, turtles, tuatara, and lizards (plus snakes and amphisbaenians, both of which appear to be evolutionary derivations from within the lizard tree), but excludes birds and mammals, both of which may have derived from within the basal group that gave rise to our "classic" reptiles (plus dinosaurs, pterosaurs, and various other extinct groups). The classes Aves and Mammalia are not sister lineages with the traditional class Reptilia. In this case, taxonomy would cause one to draw an incorrect phylogenetic tree.

Another example comes from the class Aves. Physiologists routinely compare the order Passeriformes with all other non-passerine birds (18, 81), as if the latter were the sister taxon representing one other order of birds. In reality, however, passerines are an evolutionary derivation from within other

birds, and those other birds comprise at least 22 recognized taxonomic orders (see 41, 74). Again, taxonomy misleads by implying that 23 or so orders of birds diverged simultaneously at some time in the distant past, rather than evolving by a sequence of bifurcations such that the orders themselves are hierarchically related.

Because comparative biologists have long appreciated that phylogenetic lineages may show general differences in various physiological and other traits, most of them would probably have done the sort of analysis illustrated in Figure 1B, or perhaps even further separated species into families. Nevertheless, recent conceptual and statistical developments in evolutionary biology have led to dramatic changes in the state-of-the-art with respect to choosing species for comparison, analyzing data from multi-species comparisons (including allometric relationships), and interpreting the results of statistical analyses.

The Statistical Perspective On Why Phylogeny Matters

At this point, it is necessary to take a more explicitly statistical perspective on the consideration of phylogenetic information when analyzing comparative data. First, we can ask what, if anything, a conventional regression analysis assumes about the phylogenetic relationships of the species in the data set? One might imagine that it assumes absolutely nothing, given that it makes no mention of phylogeny. In fact, however, a conventional regression analysis explicitly assumes that the species share no phylogenetic history, that they have descended from one "big-bang" speciation event at some point in the distant past. The diagrammatic representation of this assumption is referred to as a "star" phylogeny, as shown in Figure 2A. The star phylogeny is the phylogenetic translation of what conventional regression analysis assumes, as discussed in any statistics text: the data points represent a random sample from a homogeneous population, and the residuals (vertical deviations from the regression line) are independent and identically distributed.

Is it reasonable to make these assumptions when analyzing comparative data? In most cases, no. For example, when we separate the blood cell count data into their respective orders (which correspond, in this case, to separate evolutionary lineages or clades), we see a clear tendency for species to resemble other species within their own order. Moreover, only four mammalian orders are represented (e.g., no rabbits, bats or whales are included). Thus, the 146 species cannot be considered to represent a random sample of all mammals.

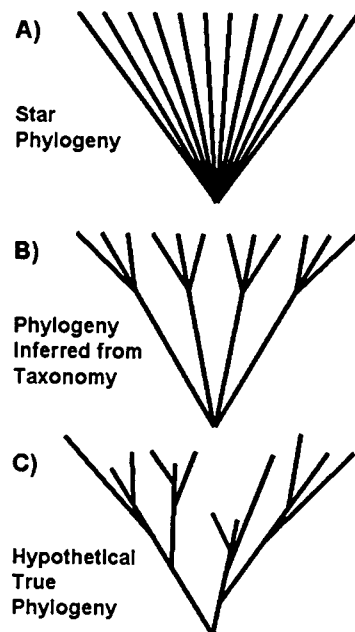


Figure 2. (A) Illustration of what conventional statistical analyses assume ("star" phylogeny) when applied to comparative data. (B) Moving beyond this, conventional statistical comparisons of taxa assume, in effect, that the taxa being compared are an unrelated series of "mini-stars" with no hierarchical structure within any taxon. assumes that the taxa actually represent separate evolutionary lineages [monophyletic groups or clades, in the language of evolutionary biology].) (C) Real phylogenies indicate hierarchical relationships and branches that do not necessarily line up along tips of the tree. Non-contemporaneous tips indicate that the rate of evolution has among branches. Real phylogenies like this cause various statistical problems, phylogenetically based statistical methods are required to analyze comparative data. Note that the horizontal axis in such diagrams is arbitrary and does not convey any information about, for example, degree of genetic or phenotypic differentiation among the species.

What are the consequences of ignoring phylogenetic relationships when analyzing comparative data? They are several and problematic, as has been demonstrated by a large number of both theoretical and empirical studies (e.g., 1, 2, 23, 32, 40, 41, 67, 73, 74, 89). First, Type I error rates will be inflated--significance will be claimed too frequently. Second, estimates of parameters, such as the slopes of scaling relationships, will be inaccurate. Third, statistical power will be affected.

The take-home message is that not attempting to incorporate phylogenetic information into analyses of comparative data is simply unacceptable. Although this message has not yet made it into some of the more mechanistically oriented fields, such as parts of comparative physiology, it is true nonetheless. I predict that in the future the use of phylogenetically based statistical methods will be as routine as the use of

statistics in general. Unlike the situation a century ago, few if any physiological studies of any kind can today be published without some use of statistics. Eventually, few if any multi-species comparative studies will be published without use of phylogenetic information. By analogy, few if any comparative studies are published today without due consideration of the possible confounding effects of body size. Today's comparative physiologists would never compare a shrew and an elephant without somehow controlling for effects of body size; in 50 years, they will never make such comparisons without attempting to control for phylogenetic effects.

The Importance of Common Rearing Conditions

Physiologists study phenotypes, which are the result of genetic effects, environmental effects, and their interactions during development and ontogeny. When species are compared, the usual presumption is that phenotypic differences among them reflect genetic differences, not just differences in the environmental conditions that they (or their mothers) have experienced. To ensure that species differences in physiology actually do reflect genetic differences, all species to be compared must be raised under common conditions (14, 15, 36, 37, 38). Unfortunately, it is typically difficult if not impossible to do so. For example, although it may be possible to capture adults (or juveniles) and to keep them under common conditions for some period of time (weeks?) prior to physiological measurement, it may not be possible to breed the animals and measure their offspring. If not, then maternal effects cannot be ruled out. Moreover, if a broad diversity of species is to be compared (e.g., including shrews and elephants), then it will simply be impossible to impose identical conditions for acclimation, let alone rearing (e.g., shrews eat animals whereas elephants eat plants).

The general rationale for including phylogenetic relationships in analyses of cross-species data sets is that genotypes and phenotypes are inherited from common ancestors. In addition, species may inherit environmental conditions (habitats and geographic localities) from their ancestors. Therefore, related species will tend to resemble each other, e.g., birds look like birds and bats look like bats. If common rearing conditions are not applied, then observed phenotypic differences among species may represent environmental effects rather than genetic differences. This would seem to weaken the specific justifications for overlaying the phenotypic data on a phylogenetic tree for analysis, but actually it would weaken any attempt to infer genetic (evolutionary) adaptation from the comparative data set. Lack of common-garden rearing conditions does not negate the importance of considering phylogenetic information, especially given that environmental

conditions (e.g., via behavioral habitat selection) may also tend to be inherited phylogenetically; rather, it weakens very generally the adaptive inferences than can be drawn from a data set. Hence, the results of any comparative study (whether of species populations) should be viewed with caution, unless it has implemented thorough common-garden controls (see also 14, 15, 36, 37, 38).

How Do We Account For Phylogenetic Relationships?

Given that ignoring phylogenetic relationships violates various assumptions of conventional statistical methods, what can we do about it? Performing an ANCOVA by order, as described above for the data shown in Figure 1, is a step in the right direction. It at least acknowledges the possibilities that orders may differ and that the within-order relationship of red blood cell count to body mass may be different (Figure 1B) from the one that appears when we ignore order as a factor (Figure 1A).

But the ANCOVA by orders is only partially phylogenetic. The analysis still assumes that each order contains no hierarchical relationships, i.e., that each can be represented by a star phylogeny, and these four stars are themselves connected as a star to the base (root) of the tree (Figure 2B). To take the analysis further and make it fully phylogenetic, we would need to specify the detailed hierarchical relationships of all 146 species.

Once we have specified the phylogeny (topology and branch lengths) of the species under study, we can employ statistical methods that use the phylogenetic information. Three main methods are available for incorporating full phylogenetic information into comparative analyses: phylogenetically independent contrasts, generalized least-squares, and Monte Carlo computer simulations. Recent work has shown that they are functionally equivalent, although quite distinct in implementation (see 41). A full description of these methods is beyond the scope of this chapter, but various reviews, with worked examples, are available (e.g., 1, 37, 40, 43, 73). In addition, free computer software is available from several sources (see Joe Felsenstein's website for a rather complete listing: <http://evolution.genetics.washington.edu/phylip/software.html>). For example, my colleagues and I have developed the Phenotypic Diversity Analysis Programs (available directly from me: see 40, 41, 43) as well as a new package named PHYLOGR (available at <http://cran.r-project.org/>).

Phylogenetic trees are estimates of (hypotheses about) the true, but unknown, evolutionary relationships of organisms. Hence, the results from any phylogenetic analysis of comparative data may be subject to modification if future information alters the arrangement of species (and possibly the branch lengths [e.g., estimated divergence times], although a

number of studies have shown that errors in branch lengths do not necessarily have fatal consequences [2, 23, 24]).

Choosing Species for Comparison

A long-standing tradition in comparative physiology (and in many other fields) has been to compare only two species, e.g., one from high altitude and the other from low, the latter offering a basis for comparison (a type of "control"). As discussed at length elsewhere, comparisons of only two species are generally inadequate for making inferences about genetic adaptation or even about mechanism (14, 15, 37, 38). However, as noted previously (37), one way to enhance the value of two-species comparisons would be to examine the results of several, perhaps completed by different workers. Indeed, as noted by Hopkins and Powell (50), comparisons of several pairs of "normal" with hypoxia-tolerant species (Canada with Bar-headed geese, Sherpa with lowland humans, mole rats with Norway rats, Green Sea with Loggerhead turtles, Mudskippers with Lungfish) all show higher P_{50} in the former of the pair. As these species pairs are only distantly related, they constitute essentially independent replicate comparisons. Thus, some sort of formal meta-analysis could be conducted to test the hypothesis that hypoxia tolerant vertebrates in general have high P_{50} . The literature probably contains data that would allow many other such paired-comparison tests (see also 32, 37).

A more common situation is that the species available for comparison are somewhat arbitrarily scattered across the phylogenetic tree. This is the typical outcome when species are chosen based on, for example, convenience, perhaps with the addition of data available in the literature. The point of this section is that the choice of particular species to be compared, whether intentional or unintentional, can have profound effects on both the utility and validity of a study. More specifically, I will argue that a phylogenetic perspective is a powerful and necessary part of choosing species for a comparative study.

Obviously, proper choice of species depends on the question asked, but determining what constitutes the best choice is actually a complicated and multi-faceted problem (e.g., 2; see also 42 pp. 437-445). For example, if one were interested in cataloging the diversity of metabolic rates across all mammals, then one would want to sample from all (or at least most) major lineages. If body size affects the trait of interest, then selecting species as different in size as possible ("mouse to elephant") is obviously an important consideration as well. Of course, actually sampling many groups may be logistically difficult as not all groups will be available either locally or through animal suppliers. Moreover, if a broad diversity of species is

studied, then it will be exceedingly difficult if not impossible to implement common rearing conditions, or even common acclimation conditions, prior to making measurements.

To study adaptation in the genetic, evolutionary sense, one first identifies an independent variable of interest, such as temperature or oxygen concentration. In general, to enhance statistical power, the species to be compared should represent a broad range of this independent variable. For example, we might study adaptation to hypoxia with a set of mammalian species (or populations), each of which occupies a fairly restricted elevational range along the continuum from sea level to very high altitude. We might then sample randomly from all terrestrial mammals. Alternatively, we might choose a particular lineage, such as rodents or primates, and sample only within that. For a given affordable sample size (number of species), the latter strategy may avoid complications caused by comparing distant relatives, which may be analogous to apples and oranges (or "chalk and cheese" in the United Kingdom)(37). In other words, distant relatives are likely to differ in many traits, not just those related to the independent variable of interest (e.g., altitude), such that a comparison of distant relatives is like an experiment with multiple uncontrolled variables.

Even within a given lineage, such as rodents, it is often possible to choose species such as to allow a sort of paired-comparisons analysis, as depicted in Figure 3C. When analyzed with phylogenetically based statistical methods, this sampling scheme actually affords higher statistical power than would be offered by the sampling scheme shown in Figure 3A (e.g., picking very distantly related species). The worst possible sampling scheme would be to choose species such that half occupied one end of the environmental continuum and the other half occupied the other end, but each of these sets of species were close relatives. When analyzed with phylogenetic statistics, this design yields low statistical power (see 40, 89).

In summary, given that one employs phylogenetically informed statistical analyses, which are necessary to guard against inflated Type I error rates and inaccurate estimates of statistical parameters (such as allometric slopes), statistical power can be greatly affected by the phylogenetic positions of the species in the analysis (89). On the positive side, choosing species so as to include multiple instances of high-low differences between close relatives (Figure 3C) can yield statistical power that is higher than that obtained by random sampling of species. Similarly, if one wishes to compare a particular species of interest (e.g., the bar-headed goose, the giraffe) with an allometric standard, statistical power can be enhanced by the use of phylogenetic methods that allow one to specify the position of the species on the phylogenetic tree (41). Thus, phylogeny can be your friend. On the negative side, however, choosing species that are phylogenetically clumped with

respect to the independent variable of interest (Fig. 3B) can lead to greatly reduced statistical power.

Recent computer-simulation studies provide additional guidance on choosing species (2). For example, the comparative physiology tradition of choosing animals that are known to be either extreme in one or more phenotypic characteristics, or that live in extreme habitats, can enhance statistical power, so long as they are not so unusual as to become a statistical outlier (perhaps because of other adaptations that we did not consider), and so long as we know where they fit on the phylogenetic tree (37).

In practice, relatively few multi-species studies in comparative physiology or physiological ecology have been designed from the ground up. Instead, organisms are included partly because they should be on statistical and/or phylogenetic grounds, partly because they are of particular (and perhaps irrational!) interest on the part of the investigator, and partly just because they were available in that part of the world (sometimes from the local pet shop). As well, many studies include data that has already been published in the literature, often by other researchers. The resulting mix of species, if analyzed in toto and by conventional statistical methods, may tend to mislead the investigator. Fortunately, if we can specify with some degree of accuracy the phylogenetic relationships of even the motleyest crew of species, then modern phylogenetically based analytical methods have great potential to "rescue" an analysis from phylogenetic obfuscation.

Finally, I would like to dispel what seems a common misunderstanding. Many biologists seem to think that if they employ phylogenetically based statistical methods most of their hard-won statistically significant results will go away. This also seems to be one reason that some workers have been resistant to their use. (Other reasons include just not wanting to be bothered with phylogeny, which is not legitimate, and lacking phylogenetic information, which is legitimate but can sometimes be overcome [e.g., see 11, 23, 24, 39].)

Biologists fear what seems to be a reduction in statistical power. However, that apparent reduction (when phylogenetically based methods are

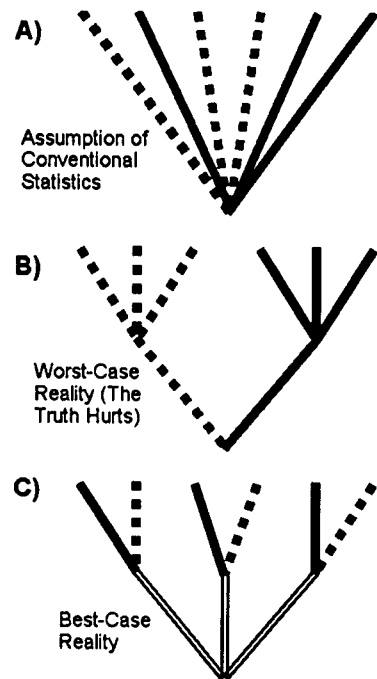


Figure 3. Three of the many possible evolutionary relationships of six species (or of six populations within a given species, e.g., *Homo sapiens*), three of which occur in one habitat type and three of which inhabit another. For example, the dashed branches might represent species inhabiting low altitudes (LA), whereas the solid branches represent species inhabiting high altitudes (HA). A goal of a comparative study might be to compare one or more phenotypes (e.g., body size, blood hemoglobin concentration) between the LA and HA species. (A) If the six data points were to be analyzed with conventional statistics, then the implicit assumption would be that none of the species share any evolutionary history beyond what they all share (i.e., since they diverged from a common ancestor). This can be termed a "star" phylogeny. (B) If the investigator were to determine the phylogenetic relationships of the six species, then they might turn out to represent just two distinct lineages, one containing all three of the LA species and the other containing the HA species. This situation might be termed "phylogenetic pseudoreplication." Instead of six independent data points, something closer to two are available. If the data were analyzed with phylogenetically correct ("PC") statistical methods, then Type I error rates could be protected to retain the a priori level for accepting statistical significance (e.g., $P < 0.05$), but the statistical power to detect a difference between the LA and HA species (if one existed) would be greatly reduced. (C) Alternatively, the evolutionary tree for the six species might be such that the closest included relative of each HA population was a different LA population. If the data were analyzed with phylogenetically based statistical methods, then not only would Type I error rates be protected, but in addition the statistical power to detect a difference between the LA and HA species would be higher than if analyzed with phylogenetically uninformed ("PU") statistics. If possible, comparative studies should be designed so that the independent variable of interest (e.g., altitude) maps onto the phylogenetic tree something like what is shown in (C), rather than what is shown in (B).

used) is illusory. Power of alternate statistical methods can only be compared when the Type I error rates of the methods are the same. Conventional statistical tests applied to phylogenetically structured data may yield seemingly higher power by allowing inflated Type I error rates. A scientist may be willing to accept statistical significance at an alpha level greater than the traditional 0.05, but he or she would want to do so intentionally and a priori, not because the species being studied were related in a hierarchical fashion. When Type I error rates are made comparable, power of phylogenetic methods can actually be higher (e.g., 67). In addition, the power of phylogenetically based statistical methods applied to phylogenetically structured data (and assuming that the topology, branch lengths, and model of character evolution are known) is actually identical to the power of conventional statistical tests applied to non-structured data (37).

Relevance to the Study of Human Altitude Adaptation

As discussed by Brutsaert (14, 15), considering the evolutionary relationships of human populations is important for understanding whether any of those native to high altitudes show evidence of genetic adaptation to high altitude. Fortunately, several human populations are native to high altitude, including some in Tibetan, South America, Ethiopia, Kenya, and Papua New Guinea. Moreover, these appear to represent at least three independent invasions of high altitude. Hence, it is possible to design a comparative study that resembles the situation depicted in Figure 3C, in which each high-altitude native population is contrasted with their close relatives that do not live at high altitude. As noted above, this sort of comparison should afford adequate statistical power for detecting altitude adaptations that actually exist, at least if the same trend is exhibited in each of the independent comparisons. Of course, the usual caveats about the need for common rearing conditions, etc., would apply, as has been discussed above and elsewhere (14, 15, 36, 37, 38).

SELECTION EXPERIMENTS

No matter how well designed, nor how interesting the study organisms, comparative studies have limitations. For example, environmental effects can be very difficult to control and may confound adaptive interpretations, especially if genotype-by-environment interactions occur. In addition, comparative studies provide data that are purely correlational. As well, they do not allow one to study evolution in action (in "real time"), nor easily to

determine the sequence of evolutionary changes that may occur during adaptation (e.g., whether behavior adapts more rapidly than underlying morphological and physiological traits [see 38, 45, 52, 55, 68]). Hence, a number of evolutionary physiologists have turned to selective breeding experiments (e.g., 10, 46, 95).

Selection experiments are one subset of approaches in the overall area of quantitative genetics (8, 14, 15, 27, 38, 45, 49, 64, 77, 78). In fact, they are the oldest "approach" in quantitative genetics in the sense that they have been occurring since humans first began developing agricultural practices, including the gradual process of domesticating various animals. Although currently less in vogue than some more recently developed types of genetic manipulations (e.g., knockouts, transgenesis), selection experiments offer a major advantage over these in being more representative of genetic changes that occur in nature in response to natural or sexual selection. In the wild, selection acts directly on complex phenotypes (e.g., life history traits, behavior), most of which are highly polygenic (affected by many genes, most of which probably have relatively small effects). Hence, allele frequencies at many loci may change in response to selection. In contrast, a typical transgenic experiment alters one or at most a few genetic loci. Accordingly, selection experiments still find an important place in the biomedical sciences (e.g., 20, 66).

At least two general kinds of selection experiments can be distinguished (38, 45, 79): artificial selection and laboratory natural selection. Traditional artificial selection involves barnyard or lab populations in which each individual in each generation is scored for some phenotypic trait or combination of traits. Some bottom or top proportion of individuals is then chosen to become the parents of the next generation. This is called truncation selection. One variation on this theme is taking at least one male and female from within each family, then allowing them to mate with other individuals in their line but outside of their own family. This is termed within-family selection, and it increases the effective population size, reduces the rate of inbreeding, and helps to eliminate maternal effects.

C. B. Lynch used within-family artificial selection to alter nesting behavior of laboratory house mice. She maintained a total of six lines, two bred randomly as controls, two selected for large nests, and two selected for small nests. Replication of experimental lines, and consistency of response, is crucial in order that ensuing differences can be attributed to the effects of selection rather than founder effects and/or random genetic drift, perhaps in combination with the occurrence of unique mutations. Lynch's overall goal has been to understand the evolution of thermoregulatory phenotypes (behavioral, morphological, and physiological), viewed as an integrated suite of interacting traits (an "adaptive syndrome"). Her selected lines have been

used in many subsequent studies, and informative parallels have been drawn with clinal variation in wild (introduced) populations of house mice in North America (16, 61, 62, 63).

In laboratory natural selection, freely breeding populations are exposed to altered husbandry conditions, which could favor altered demographic schedules, or to altered environmental conditions, such as different temperatures. An example of this approach is Barnett and Dickson's experiments in which wild house mice were captured and used to establish two breeding colonies, one housed at approximately room temperature and the other in the cold. They performed two such experiments, for 9-14 generations, once in Scotland (7: average room temperatures of 21 and -3 C) and then again in Australia (23 and +3 C) (4, 5). In both experiments, various changes were observed, at least some of which seemed to represent the evolution of genetic adaptations to the cold (6). The results were rather complicated, however, and in both cases only a single line was kept in either the "control" (room temperature) or "experimental" (cold) condition. This lack of replication makes it difficult if not impossible to determine with confidence whether apparently adaptive changes are really so (i.e., the result of an altered selective regime) or the result of random genetic drift.

Artificial Selection for High Wheel Running in Mice

Our laboratory has conducted an artificial selection experiment to increase levels of voluntary wheel-running behavior in mice, and we are monitoring correlated changes in other behavioral, morphological, physiological, and biochemical traits. The overall goal is to understand how increased activity evolves, at levels ranging from motivation to exercise physiology (56, 57, 51, 75, 83, 84, 85, 86)

The original progenitors were outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain, purchased from Harlan Sprague Dawley in 1993 (83). Genetic variation in the base population is similar to variation among individuals in wild populations of *Mus domesticus* (19, 76; and references therein). After two generations of random mating, mice were randomly paired and assigned to 8 closed lines (10 pairs in each). In each subsequent generation, when the offspring of these pairs were 6-8 weeks old, they were housed individually with access to a running wheel for 6 days and a computer recorded wheel revolutions in 1-min intervals (1.12-m circumference, attached to standard clear plastic housing cages via a stainless steel tube inserted into a hole in the wall of the cage). In 4 "selected" lines, the highest-running (quantified as total number of revolutions run on days 5 and 6 of the six day test) male and female from each family were chosen as breeders to propagate the lines to the next

generation. In the 4 "control" lines, a male and a female were randomly chosen from each family. Within all lines, the chosen breeders were randomly paired except that matings between siblings were disallowed.

The purpose of maintaining replicate selected and control lines is to account for random genetic changes, such as founder effects and drift, which can cause lines to diverge even in the absence of selection. Any particular genetic or phenotypic difference between a given selected line and a given random-bred control line may or may not be related to the phenotype that was actually under selection. Inferences about the causal factors underlying phenotypic changes in a selected line are greatly strengthened if replicate lines are maintained (48).

After 16 generations, revolutions/day had increased 2.7-fold (mainly by increased running speed) and reached an apparent selection limit (Figure 4 shows data through 24 generations). This limit appears to correspond to the maximal aerobic speed estimated in the base population (56), and neither maximal oxygen consumption, when measured a week prior to wheel testing, nor basal metabolic rate has responded to selection by generation 22 (unpublished results). (Maximal oxygen consumption may show differences between the selected and control lines at other ages and/or under different housing conditions (84).)

Both sexes, but especially, females, have primarily increased their average running speed rather than the amount of time spent running (Figure 5: see also 56, 75, 83). Various morph-physiological differences between selected and control lines exist, some of which may represent genetic adaptations for sustained exercise (44). For example, mice from selected lines have higher insulin-stimulated glucose uptake in some hindlimb muscles (26) and more symmetrical hindlimb bone lengths (T. Garland and P. A. Freeman, unpublished results). Selected-line mice are smaller in body mass (85) and have less body fat than controls, at least under some conditions (86). When housed with vs. without access to running wheels for 8 weeks, suborganismal training responses (e.g., increases in hematocrit, citrate synthase activity of hindlimb muscle) are often greater in mice from selected lines (genotype-by-environment interaction), presumably because they run more (51). Motivation is now under study, and pharmacological experiments suggest altered dopaminergic function in the brains of selected-line mice.

Aside from the above-mentioned consistent differences between the selected and control lines, the four replicate selected lines show statistically significant differences in a number of traits, including wheel running itself. Of particular interest, two of the four selected lines now contain a high frequency (approximately 50%) of individuals with small muscles, in which the gastrocnemius exhibits an almost 50% reduction in mass, along with an

approximate doubling of mass-specific oxidative capacity (44). Comparisons of parents and offspring suggest that this phenotype is inherited as an autosomal recessive allele (unpublished observations). Moreover, population-genetic model fitting (in collaboration with Martin Morgan and Patrick A. Carter) provides evidence that the allele must have been under positive selection in the two selected lines. (Presumably, the other two selected lines lost the allele, which was rare in the base population, by chance either at founding or shortly thereafter by genetic drift.) Our working hypothesis is that these "mighty mini-muscles" are adaptive for sustained, relatively high-speed running, perhaps because of shorter diffusion distances. In collaboration with Helga Guderley and Philippe Houle-Leroy, we are now testing this possibility.

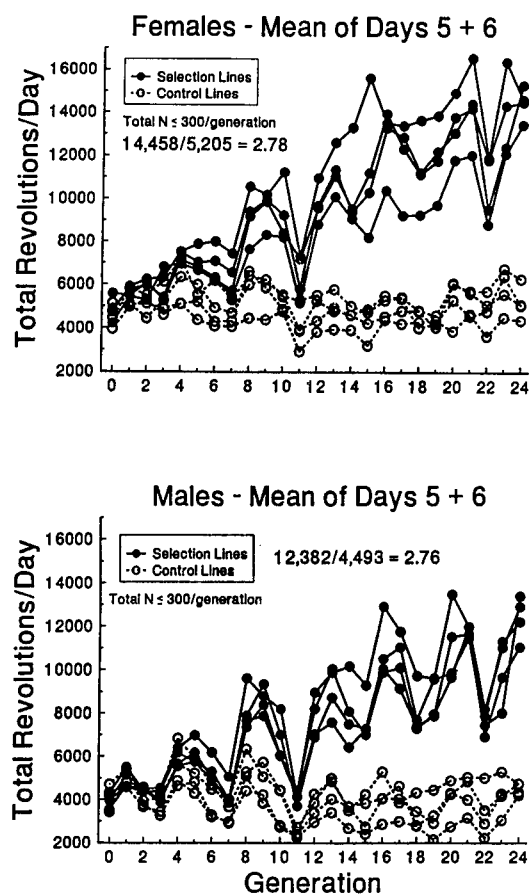


Figure 4. Wheel running (line means) of 8 lines of house mice either selected for high wheel running or bred randomly as controls. Dips in wheel running that seem to occur approximately every four generations (especially notable in males) correspond to summer generations, during which elevated humidity (and sometimes temperature) may cause reduced activity. Note that females always run more than males, but that the response to selection, relative to control lines, is similar in the two sexes.

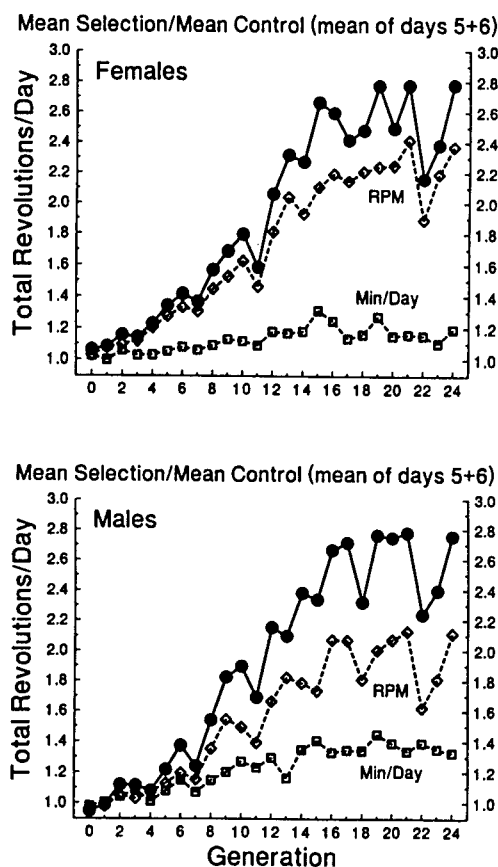


Figure 5. Ratio of mean wheel running for the selected compared to the control lines.

Mice in the selected lines, especially for females, have accomplished more total revolutions (closed circles and solid line) per day mainly by increasing their average running speed (RPM, calculated as total revolutions divided by the number of 1-min intervals during which any revolutions were recorded), rather than the number of minutes spent running (Min/Day).

Prospects for the Use of Selection Experiments to Elucidate Adaptation to Hypoxia

Applications of selection experiments to the study of hypoxia adaptation are easy to envisage. For example, in the mode of laboratory natural selection, one might establish replicate lines of mice or rats at high altitude or in hypobaric chambers, while also maintaining control lines at sea level. The mice could be allowed to breed freely (within lines) for perhaps 10 generations (as in Barnett's studies of cold adaptation in mice: 4, 5, 6, 7). As generations passed, various other candidate phenotypes (traits thought to enhance physiological function under conditions of low partial pressure of

oxygen) could be monitored, such as hemoglobin levels, shape of the oxy-hemoglobin dissociation curve, lung capacity, pulmonary diffusing capacity, metabolic rate or activity levels.

Alternatively, one could implement selective breeding. Lines could be established in any convenient animal quarters. An expeditious test of whole-animal hypoxia tolerance could be devised, such as time to lose the righting response when the partial pressure of oxygen is lowered acutely. The most tolerant individuals would then be chosen as parents to produce the next generation. Again, candidate phenotypes would be monitored, i.e., subordinate traits hypothesized to facilitate hypoxia tolerance.

In either type of selection experiment, it would be predicted that one or more key phenotypes would change consistently in the selected lines (e.g., hemoglobin levels). Most likely, this would represent a trait that was somehow limiting to hypoxia tolerance, or at least to optimal function under hypoxia. If one or more traits did change consistently, then they would represent putative adaptations for hypoxia.

Of course, it is also possible that replicate lines will show a similar overall response, e.g., at the whole-animal level, but that the details of underlying adaptive mechanisms will differ, as appears to be the case in our replicate lines of house mice that have been selected for high wheel running. Interestingly, Tibetan and Andean human populations show what appear to be different adaptations to high altitude -- if, in fact, they are adaptations (see 14, and references therein)!

In any case, mechanisms can be complicated and slippery things. Therefore, one might subsequently propose to do the reciprocal experiment (see 38). That is, one could establish new lines, from the same original base population, and select directly on the putative adaptation. Thus, one might select directly for higher hemoglobin levels (mice have been successfully selected for hematocrit [80]). If hypoxia tolerance then increased across generations, then this would strengthen the original interpretation that elevated hemoglobin levels were an adaptation for hypoxia. Finally, as noted previously (38), selection experiments could be used to test predictions of symmorphosis (25, 35, 91, 92), i.e., that multiple components of a physiological pathway should be matched in capacity, and hence that they should all change approximately in parallel when selection is imposed at the level of organismal performance.

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Chapter 10

Genetic and environmental adaptation in high altitude natives

Conceptual, methodological, and statistical concerns

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Abstract: A great number of physiological and anthropological studies have investigated Andean and Himalayan populations native to high altitude (HA). A non-scientific survey of the extant literature reveals a relatively liberal tradition of inferring genetic (evolutionary) adaptation to HA in these groups, often based on limited evidence and/or based on study designs insufficient to fully address the issue. Rather than review the evidence for or against genetic adaptation, and in order to provide some perspective, this paper will review relevant conceptual, methodological, and statistical issues that are germane to the study of HA native human groups. In particular, focus will be on the limitations of the most common research approach which bases evolutionary inference on the comparison of phenotypic mean differences between highland and lowland native populations. The migrant study approach is discussed, as is a relatively new approach based on genetic admixture in hybrid populations.

Key words: natural selection, developmental adaptation, acclimatization, hypoxia, admixture, migrant study

INTRODUCTION

Since the pioneering studies of Andean natives by Carlos Monge and Alberto Hurtado in Peru in the middle 1900s, research has focused on the phenotype of highland native populations worldwide. By phenotype is meant the outward appearance of an organism as a result of both genetic and environmental effects, as originally defined by Johannssen (71). This is not say that molecular genetic or serological studies haven't been conducted in an attempt to understand the genetics of highland populations (e.g. see the

early studies in the Andes (36, 37, 83, 84) and the more recent studies using a candidate gene approach (105, 106)), but rather that most of what we think we know about genetic adaptation to high altitude (HA) comes from the analysis of phenotypic differences between highland and lowland groups. In fact, at present, no population specific alleles or allele frequencies have been described to show genetic adaptation in any highland group. Thus, researchers wishing to address the question of evolutionary adaptation to HA are left to separate genetic from environmental factors, a difficult task at best given that these factors interact to determine the phenotype. However, there is an advantage of HA studies in this regard as historical processes of population migration and admixture provide a model system uniquely suited to this type of analysis. In this paper, I will focus on studies that have been applied using phenotypic comparisons between groups, but I will not be reviewing explicitly the evidence for or against genetic adaptation as this question has been addressed in a number of past reviews (2, 4, 28, 62, 87, 90-92, 102). Rather, I will emphasize analytic and statistical issues that potentially limit evolutionary inference via phenotypic comparison. In addition, I will describe a unique migrant/admixture study design that has not yet been applied to the question of genetic adaptation, but which may provide benefits over previous study approaches.

Focus On The Phenotype

There is no doubt that highland populations worldwide are phenotypically unique. For example, the archetype "highland native phenotype" is a large chest dimension and/or lung volume. Figure 1 shows vital capacity (FVC) as a function of stature from populations worldwide residing above 3,000 meters. Included are a number of migrant European populations from North and South America who are native to HA in the sense that they were born and raised above 3,000 meters. FVCs in Andean, Himalayan, and European populations born and raised at HA all exceed sea level standards by up to 30%. The Ethiopian highlands have received little attention, and the two populations represented in Figure 1 (who fall within sea level norms) may not have been representative samples. In addition, these two populations have larger FVCs compared to lowland groups in Ethiopia (60), so it is not clear if the US reference standard applied is appropriate. Other frequently discussed highland native phenotypes include larger birth weight babies (57, 90, 117), greater physical work capacities (19, 41, 110), higher arterial oxygen saturations (SaO_2) at rest and/or during exercise (5, 17, 109), smaller alveolar-arterial oxygen gradients during exercise (30, 120), and a metabolic organization that is both more efficient and which favors carbohydrate utilization over fatty-acids (61, 67, 82). The extent to which there is data to support genetic adaptation based on any of

these phenotypes ranges from very good (birth-weights and lung volumes) to unclear (exercise and metabolic phenotypes) (see ref. 18 for a discussion of the VO_2max phenotype).

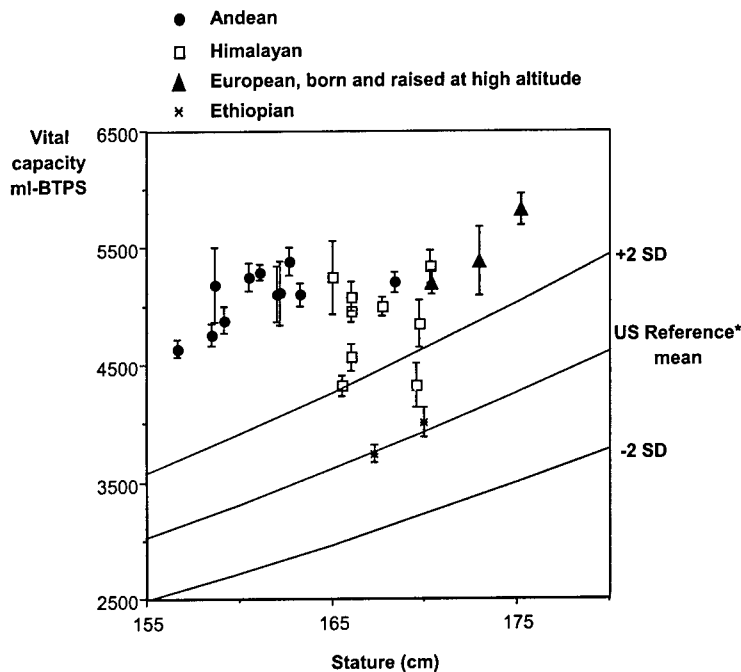


Figure 1. Mean forced vital capacity \pm standard error in adult males as a function of stature from studies of highland native populations worldwide. Data to construct the figure come from the following sources. Andean natives (16, 18, 40, 52, 68, 70, 73, 109, 116). Himalayan natives (26, 31, 45, 46, 110, 116, 119, 120). Ethiopian natives (60). Europeans born and raised at high altitude (18, 29, 40). *US reference mean values by stature from Polgar and Weng (100).

Phenotype focused studies fall into two broad categories, those which apply the statistical techniques of quantitative genetics and those which compare mean values of complex phenotypes between different population groups. While both approaches may have the goal of partitioning genetic from environmental variance in the analysis of a phenotype, this is more explicitly the goal of quantitative genetics.

Quantitative genetics

The basic goal of quantitative genetics is to analyze the amount and nature of genetic variation *within* a population for a continuously varying phenotypic trait, and to partition total phenotypic variance (σ^2_P) into genetic (σ^2_G) and environmental (σ^2_E) components where $\sigma^2_P = \sigma^2_G + \sigma^2_E$ (34, 81).

The proportion of the total phenotypic variance that is due to genetic differences between individuals is described by a population summary statistic termed *heritability* (h^2), where heritability in the *broad sense* refers to the proportion of phenotypic variance attributable to all genetic effects (i.e., σ^2_G / σ^2_P), and where heritability in the *narrow sense* refers to the proportion of phenotypic variance due to additive genetic effects alone (i.e., σ^2_A / σ^2_P). Variance partitioning in this manner was originally of interest to plant and animal breeders, but quantitative genetic analysis was embraced by evolutionary biologists in the 1970's and 1980's (81), and has recently been applied with great promise in both the Andes and Himalayas (5-7).

Many studies show significant heritabilities for important highland phenotypes, including measures of chest dimension, pulmonary function, hemoglobin concentration, resting ventilation, and resting SaO_2 (5-7, 79, 94). Beall's studies in the Himalayas, in particular, make clear the great potential of this approach. In segregation analyses based on large scale pedigree studies of native Tibetan groups, Beall et al. give statistical evidence for a major gene explaining a large proportion of the variance in resting SaO_2 (6, 7). This putative dominant allele is said to exist at ~45% frequency and to confer a 5-6 percentage point higher resting SaO_2 . This is an important result given the functional importance of SaO_2 , but limitations of the approach should also be clarified. As a statistical result, there is no information on the genetic architecture of the trait, including the mode of inheritance, the chromosomal locations of the putative gene, gene products, functional effects of genes on the phenotype, and the pattern of underlying genetic variation affecting the trait between population groups. The latter is particularly important and emphasizes the fact that quantitative genetic analysis takes place *within* rather *between* population groups of interest. This was recently made clear when similar analyses in the Andes showed no additive genetic variation in resting SaO_2 in Aymara (5). Nevertheless, quantitative genetic studies can point the way to an underlying genotype. Segregation analyses can be used to determine the presence of major genes (11, 12, 33), and linkage mapping can be used thereafter to determine the approximate chromosomal locations of genes using a library of human genetic marker loci termed quantitative trait loci (QTLs) (13, 104). The general strategy has been usefully applied to the study of many complex disease phenotypes, leading, for example, to identification of BRCA "breast cancer genes" on chromosome 17q encoding for tumor suppressor proteins (58, 77, 80, 121). The BRCA genes have now been examined at the population level revealing relatively high frequencies of deleterious alleles in Ashkenazi Jewish and Belgian populations (8, 48).

Comparison of complex phenotypes between different population groups

Studies that rely on the comparison of mean values for complex phenotypes between populations with differing histories of exposure to HA comprise the great majority of studies that one encounters in the anthropological and physiological literature. Among these studies it is relatively easy to find inference to genetic adaptation vis-à-vis highland native groups (1, 2, 6, 15, 18, 32, 40, 41, 45, 50, 54, 56, 61, 62, 66, 69, 75, 76, 89, 95, 96, 98, 99, 105, 108, 112). The strength of inference depends on a number of statistical and methodological issues not fully appreciated by all.

The source problem is that phenotypic comparisons between human populations are *observational* rather than *experimental* studies. Figure 2 may help to clarify some of the analytic issues that result from this fact. Panel A shows the paradigm of an experimental study. An experimental study is initiated by sampling at least two groups from a homogenous source population. One group receives a treatment while the other serves as a control. Statistical testing for a treatment effect proceeds based on established criteria for significance i.e., $\alpha=0.05$. However, this does not represent the reality of a human population based study which is given on the right. While all human populations were certainly derived from the same source population at some time in the distant past, the treatment of interest (i.e., hypoxia) was not applied historically until after considerable time elapse. Thus, at the time of the treatment, study groups are likely to have differed already (due to previous natural selection, genetic drift, mutation etc...). Group differences may be in the direction of the study hypothesis (assume a 50% probability) which means that the alpha-level used in support of an evolutionary hypothesis is likely to be much higher than 0.05. Garland et al. have discussed this issue at length in the physiological ecology literature (44). For human studies, the implication is that many phenotypic differences (between highland and lowland groups) exist *a priori*, and any inference to natural selection should be carefully evaluated, even if the difference can be rationalized based on potential benefit to life at HA.

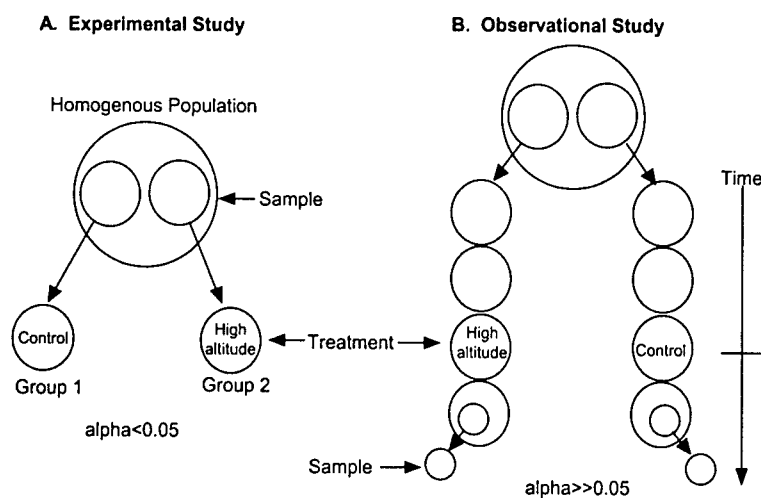


Figure 2. Experimental versus observational study approaches. This figure is based in part on a description of statistical problems related to the comparison of only two populations given in Garland and Adolph (44). See text for details.

In addition, considerable time has also elapsed since the “imposition” of the treatment. Various estimates place Tibetans at HA from 7,000-50,000 years ago (23, 118), while Andean groups may have resided at HA for as long as 10,000 years (20). The normal caveat about whether selection pressure was constant over the duration of exposure certainly applies, but more important are historic demographic effects that may have affected population structure over the time since exposure. For example, in the Andes, Spanish conquest 400 years ago led to the decimation of indigenous populations by as much as 95% of pre-conquest density (25). Were the Ameridians who passed through this historic bottleneck in fact a representative sample of those who experienced natural selection during the preceding generations? If much of the decimation was due to infectious disease, then we would not expect a representative sample, at least not for those genetic systems related to disease immunity e.g., the human leukocyte antigen (HLA) system. In addition, admixture between European and Amerindian populations has been widespread since the Spanish Conquest, with significant European admixture into the Amerindian gene pool (21). The basic analytic model of phenotypic comparison is weakened (or even invalidated) with respect to evolutionary inference if simple assumptions like these are not addressed.

The observational approach also suffers in that study independent variables may be confounded. This is best illustrated by example. Figure 3 shows maximal aerobic capacities (VO_{2max}) as a function of stature in highland native populations worldwide, including Europeans born and raised

at HA. These VO_2max values have been corrected for the altitude at which they were measured. One strategy to interpret variability and to isolate genetic adaptation is to compare Andean and Himalayan populations with populations having experienced the same environment during the growth and development period (see migrant studies below). However, it is not clear what a straight forward comparison of VO_2max (lmin^{-1}) would yield given the differences in body size and body composition between populations (see Fig. 3). Further, consider that there are large differences between populations in daily physical activity. Andean natives engage in strenuous subsistence activities with high rates of daily energy expenditure which clearly must impact aerobic capacity (74). Thus, for this one phenotype, both body size and physical activity pattern are nearly perfectly confounded with the main study independent variable, ancestral group (19, 41). Other phenotypes of interest may be similarly confounded. For example, birth-weight is affected by maternal nutritional status (55). Resting ventilation, SaO_2 , and muscle metabolic/morphologic characteristics are all affected by physical activity patterns (47, 107, 114). Hemoglobin concentration is affected by iron nutrition (3). And, the prevalence of chronic mountain sickness may depend on diagnostic criteria or accessibility to health care that can differ between populations (88, 99)

There is another class of environmental effects that also limit the ability to infer genetic adaptation based on a comparison of phenotypes between different population groups. Often investigators fail to account for environmentally induced plasticity in the phenotype which may occur over the short term (acclimatization), over the growth and development period (developmental acclimatization), or over intergenerational time (maternal effects). For example, mammals possess a homeostatic "program" that ensures a ventilation increase on initial hypoxic exposure, reflecting an ancient acclimatization response also common to birds (10, 113). Continued hypoxic challenge *modifies* (increases) erythropoietin (EPO) gene transcription in humans resulting in a higher hemoglobin concentration over time (14). Over the course of the growth and development period, developmental acclimatization occurs when environmental effects modify gene transcription and/or translation to irreversibly affect the phenotype (38, 39). As Figures 1 and 3 make clear, both lung growth and aerobic capacity

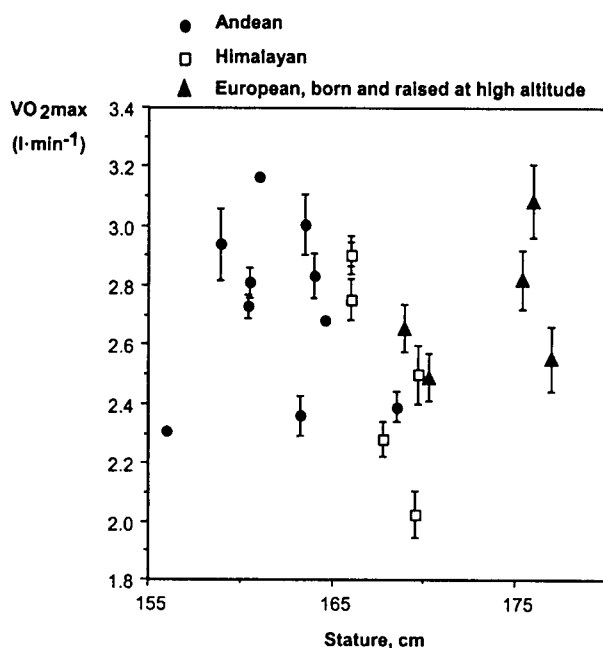


Figure 3. Mean maximal oxygen consumption \pm standard error ($\text{VO}_{2\text{max}}$, $\text{l}\cdot\text{min}^{-1}$) in adult males as a function of stature from studies of highland native populations worldwide. Data are from the following studies where it was possible to obtain absolute $\text{VO}_{2\text{max}}$ values ($\text{l}\cdot\text{min}^{-1}$) and stature. Andean natives (19, 35, 41, 42, 51, 78, 85, 86, 109, 111). Himalayan natives (26, 45, 46, 97, 110). Europeans born and raised at high altitude (19, 30, 41, 53, 86). In three studies variance about the mean could not be determined.

are affected by early life hypoxic exposure (15, 18, 40, 49, 72). Finally, maternal or paternal *non-genetic* factors may influence offspring phenotype, encompassing a category of environmental effects known collectively as “maternal effects” (9, 93). A well known maternal effect is the intergenerational effect of poor maternal nutritional status on infant birth-weight and subsequent stature as an adult (101).

Through maternal effects, there is the potential for *non-genetic* adaptation to HA over intergenerational time. Consider the scenario of a lowland migrant woman giving birth at HA. Birth-weights are considerably reduced in lowland migrant populations to HA due to the stress of hypoxia (90). First generation infants are clearly not provided with an optimal in-utero environment, but this may improve due to developmental responses accrued each generation. In this sense, maternal effects may take a number of generations to “wash out”, and such effects have confounded previous plant and animal breeding studies (9, 43).

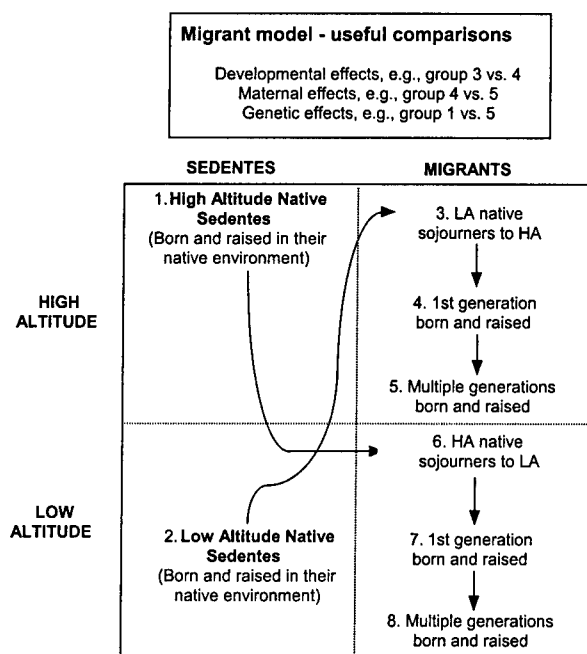


Figure 4. A complete migrant study design (adapted from (59)) includes highland and lowland native populations in their native environments (sedentes, groups 1 and 2) and migrant populations in a non-native environment. Migrants may be recent sojourners, 1st generation migrants born and raised in a non-native environment, or multiple generation migrants who are the children or grandchildren of previous migrant groups. Comparison of mean phenotypic values between specified study groups may give information about developmental, maternal, and genetic components of high altitude adaptation.

The traditional approach to partitioning out environmental sources of phenotypic variance has been to use a migrant study design as described in Figure 4 (59). This approach is conceptually similar to the experimental *common garden* study developed in quantitative genetics (24). These studies which have so far only been conducted in the Andes are logistically difficult and require large sample sizes which has likely limited their application only to the study of phenotypes that are easy to measure such as aerobic capacity, pulmonary function, and birth-weight (17-19, 40, 41, 56). In practice, migrant studies are problematic to interpret. Most do not explicitly address the issue of maternal effects, often it is not clear if migrant populations are representative of their source populations, admixture is usually an issue, and confounding is an ever-present possibility.

Some related studies have investigated highland natives migrant to the lowlands (18, 63-65, 67, 69, 73, 82, 98), or highland natives born and raised in the lowlands (18, 75, 76, 112). For these studies it may be erroneous to assume that a given trait is a genetic adaptation either (1) because it does not

de-acclimate at sea level (67, 98), or (2) because it is present even in the lowland born children of highland natives (75, 76). In particular, traits that do not de-acclimate may represent ontogenetically fixed developmental adaptations, and traits which persist in the lowland born children of highland natives may represent maternal effects.

Admixture based studies

A very few studies in highland native populations have used an admixture-based approach in order to assess genetic adaptation (27, 40, 41, 50). There is a great appeal to such studies because the fundamental reality of population admixture is recognized (and exploited) rather than ignored or assumed negligible. The studies of Greksa and Frisancho both used skin reflectance as an independent variable to assess the degree of European admixture in Mestizo populations in Bolivia. In both studies, skin reflectance was negatively correlated to measures of lung volume, where individuals with low skin reflectance (i.e., a high proportion of Amerindian genes) had larger lung volume measures. While confounding may still be an issue using this approach, the potential is greatly reduced given the single population group as the focus of investigation. Sample size requirements may also be reduced compared to the migrant approach.

Unfortunately skin reflectance is a quantitative trait which does not yield valid estimates of individual admixture, only valid estimates of group level admixture (103). Individual admixture is properly estimated by the use of serological or DNA level genetic markers. In the past only a few informative marker systems were available, but today libraries of autosomal, mitochondrial, and y-chromosome markers can be applied for admixture analysis between different population groups. In principle, admixture produces gametic association between linked loci (linkage disequilibrium) as a function of genetic difference between parental populations and admixture rate (22). Linkage disequilibrium is used to map genes for complex phenotypes that are different between two parental populations. For example, a recent study using the approach shows a genetic component to the high prevalence of non-insulin dependent diabetes mellitus (NIDDM) in an admixed U.S. Pima Indian population (115).

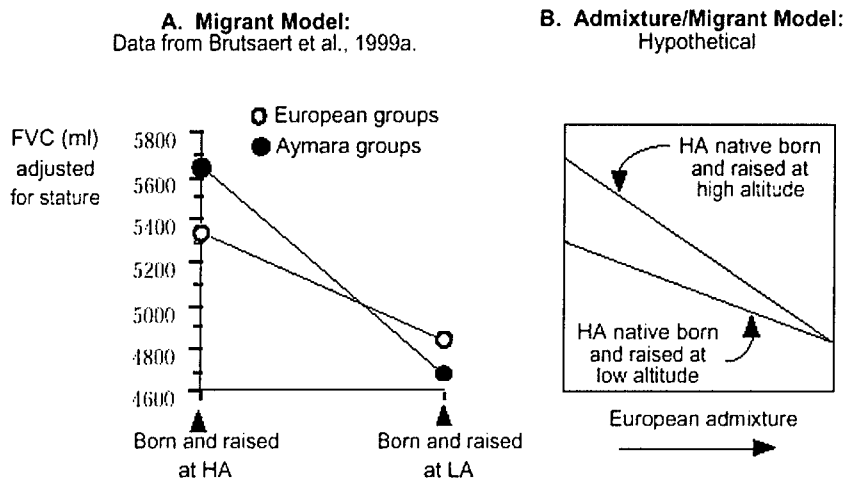


Figure 5. The migrant study design versus admixture/migrant hybrid design. Panel A: data from Brutsaert et al. (18) suggesting gene-by-environment interaction. Panel B: hypothetical model where phenotype is expressed as a function of individual admixture in groups of highland natives born and raised at high and low altitude. See text for details.

Figure 5 illustrates certain advantages of the admixture based approach as it might be used in order to assess gene-by-environment interaction in the determination of HA native phenotype. Panel A gives stature adjusted FVC mean values measured in four different study groups as part of a migrant study conducted in Bolivia (18). This was a “complete” migrant study in-so-far as data were obtained from Aymara and European groups in both their native environments and also as migrants born and raised in a non-native environment. Aymara migrants born and raised near sea level do not have larger FVCs compared to sea-level natives, but Aymara at HA do have larger FVCs compared to Europeans born and raised at HA. Thus, these data suggest that the Andean phenotype depends on an interaction of the Andean genotype with developmental exposure to hypoxia. Of course, this is a tenuous interpretation given the many problems discussed above. In particular, there are confounding issues to consider. For example, it is unclear how to normalize lung volume for body size given the large differences in stature (more than 12 cm) between these two study groups.

Panel B suggests an alternative using a combination of both the admixture and migrant study approaches. The advantages of this design are principally related to the issue of confounding, but smaller samples sizes and logistical ease may also be possible benefits. In the Andes, admixed European/Ameridian populations are widespread, both in the highlands and in the lowlands. Most lowland populations are recent migrants (within the

last 50 years) to urban centers such as Santa Cruz in Bolivia (400 m), and Lima in Peru (sea-level). Importantly, highland and lowland populations are relatively homogenous with respect to socioeconomic and lifestyle considerations. The same approach might be used in the Himalayas where admixed Tibetan and Han Chinese populations have already been studied at the group level (27), and where similarly admixed populations may exist at lower altitudes in Kathmandu, Nepal (1,300 m)(75, 76). Even if admixed populations are studied at HA only, precluding the possibility of testing for gene-by-environment interaction, the approach affords researchers the opportunity to explicitly address the issue of genetic adaptation. Further, the approach may allow the investigation of phenotypes so far not studied due to the logistical challenges of the migrant approach.

SUMMARY

In this paper I have presented a number of conceptual, methodological, and statistical issues that should be carefully considered when evolutionary inference is made based on phenotypic mean comparisons between lowland and highland native groups. While highly problematic, the strategy of comparing phenotypes to isolate genetic adaptation has not been fully exploited e.g., consider the admixture/migrant study design described above. Further, the relevance of continued study at the phenotypic level (even in the face of rapid advances in molecular biology) should be emphasized. Natural selection works on the phenotype only. Therefore, if we are to take an integrative approach, the effect of the gene must be evaluated with respect to the way that it interacts with the environment to produce a beneficial phenotype.

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Chapter 11

Common themes of adaptation to hypoxia

Insights from comparative physiology

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Abstract: Many vertebrate animals have superior tolerance to environmental hypoxia compared to humans. For example, turtles tolerate an environment of 100% N₂ for several hours, without apparent ill effect. This hypoxia tolerance is not limited to heterotherms, as some species of marine mammals, such as the northern elephant seal, may voluntarily dive for periods of up to 2 hours. Torpid bats exhibit prolonged periods of apnea and passive diffusion of oxygen down their trachea through an open glottis supplies a significant amount of the oxygen uptake. The Ruppell's griffon holds the known avian record of flight at 11,278 m, and other birds regularly migrate at altitudes over 8000m. These animals exhibit diverse adaptations for tolerating their hypoxic environment, many of which are poorly understood. Some of these strategies include 1) the ability to lower metabolic rate when exposed to hypoxia 2) the ability to recruit alternate biochemical pathways for energy production 3) a left shifted oxy-hemoglobin dissociation curve 4) more efficient pulmonary gas exchange 5) the ability to alter blood flow distribution under hypoxic stress. Although there are common themes of animal adaptation to hypoxic stress, many animal solutions are unique.

Key words: hypoxia tolerance, anoxia, gas exchange, comparative physiology, P₅₀

INTRODUCTION

Oxygen supply for some animals may vary within hours from an ambient partial pressure for oxygen (PO₂) ranging from almost complete anoxia to ~500 Torr (26). In addition the range of oxygen demands which vertebrates must meet is huge, and may vary within a single animal up to 1000 fold during different states such as torpor and exercise (50). Oxygen demands

also vary between animals of different size, and depending on their degree of body temperature regulation and mode of locomotion.

Evolutionary solutions to the oxygen supply and demand problem have produced diverse strategies, many of which are unique to a particular species. However some traits, such as the leftward shift of the oxygen-hemoglobin dissociation curve (see below) appear to be relatively robust. Comparative studies may offer valuable insights into hypoxia tolerance, and comparisons across several species can provide information about how design effects function. For example, as lungs evolved to support homeothermy and meet greater metabolic demands, diffusing capacity was increased by subdividing the lung into smaller gas exchange units. Possibly the "benefit" of increased diffusing capacity is greater than the potential "risk" of increased ventilation-perfusion inequality between gas exchange units.

However, caution must be exercised in attributing "adaptive significance" to a physiological characteristic, when using a comparative approach, especially when viewed from a mammalian viewpoint. Using lungs as an example, these organs have evolved for many non-respiratory functions, particularly in non-mammalian species. Aquatic reptiles must adjust lung volume to achieve neutral buoyancy during surfacing or diving, when avoiding predators, or pursuing prey (38). Reptilian lungs and avian air sacs serve social functions by inflation during sexual displays, acting as resonators for mating calls (14) and some lizards can inflate their apparent body size by 40% or more when threatened by an enemy (38). In addition, evolution does not operate on a "clean slate" and lung designs in modern species reflect their ancestry. Many lung designs might satisfy the respiratory needs of relatively primitive organisms which may not require the exquisite balance between O₂ supply and demand, for example, that a modern homeotherm requires. Therefore a variety of lung structures might have satisfied the respiratory needs of primitive vertebrates in oxygen rich environments, and the diversity of lung structure and complexity in modern animals may reflect aspects of evolution that have nothing to do with respiratory adaptations *per se*. However, keeping these constraints in mind, it is possible to evaluate high performance species to glean insights into the mechanisms of tolerance to extreme environments, especially when traits appear across very diverse species.

Hypoxia tolerant species

It is common to think of hypoxia tolerant species as those that reside at or travel regularly to high altitude. Of these the bird is undoubtedly the high altitude champion. Information on high altitude flight records is often gained when the unfortunate bird has collided with commercial aircraft, although

some reports arise from sightings made by high altitude climbers. The bar headed goose (*Anser Indicus*) migrates from a winter site on the Indian subcontinent to breeding grounds in the South central regions of Asia. These animals fly non-stop from essentially sea level over the Himalayan mountains before nesting at 5,500m (49). However, the all time record for high altitude flight is held by an Old World vulture, the Ruppell's Griffon (*Gyps rueppelli*) who was sucked into a jet engine at 11,278m (37,000 ft) in 1973 (30). By contrast, only extraordinary humans can barely stagger to the top of 8,000m peaks and there is evidence to suggest that those who do so may suffer minor, but detectable, neuropsychological deficits (25).

Especially compared with birds, bats, the only true flying mammal, are not reputed to be high altitude fliers. Experimentally, they are remarkably hypoxia tolerant, and survive acute decompression to simulated altitudes of 11,000m (55). One possible explanation for lack of high altitude flight on the part of bats, may be convective heat loss from the large surface area of the wing, which unlike the bird wing contains locomotor muscles and lacks insulation.

Several mammals are noted for their high altitude residence and hypoxia tolerance, including llama and alpacas, and several rodent species. The rodents are of special interest because they demonstrate differences between low altitude and high altitude animals both between species and within species. For example *Chinchilla lanigera* lives from sea level to 2,000m in the Andes and *Chinchilla brevicaudata* lives between 2,000 and 5,000 m altitude in the same range (15). Similar partitioning of altitude niches exists between North American species of squirrels (10). In contrast, the deer mouse (*Peromyscus maniculatis*) is probably the most widely distributed mammal in North America and occurs from below sea level in Death Valley, California to the highest altitudes in the lower forty-eight United States at over 4,300m (10). Furthermore, there are significant differences in exercise capacity and physiological O₂ transport between low and high altitude populations of deer mice that have a genetic basis (11). A potentially valuable model for future studies is the house mouse (*Mus domesticus*). This species occurs in nature over a wide range of altitudes, for example from sea level on the South American coast to over 4,000m in Andean copper mines. Because this species did not occur in South America before the arrival of Spanish explorers, boundaries can be placed on the time for any adaptations to altitude that may be discovered now. Studies of such wild house mice can be complemented by laboratory studies on the same species, which has been inbred for research. The power of such comparative studies is enhanced by the modern tools developed to study mouse genetics, such as "knock-out, knock-in and knock-down" mice, DNA microarrays and, very soon, the whole mouse genome.

Fresh-water fish occur over a wide range of altitudes, but many of the species commonly studied (e.g. trout) have been introduced to high altitude

waters by humans at varying times. Considering the relatively low O₂ capacity for water compared to air and effects of temperature on the O₂ capacity of water, one might not expect changes in selective pressure on fish to be as large over an altitudinal gradient as it would for air breathers. Studies of trout living at different altitudes have not demonstrated convincing adaptations to hypoxia that could be distinguished from differences explained by temperature or food availability (8). Daily and tidal fluctuations experienced by fish at sea level provide more interesting data on adaptations to hypoxia as discussed below.

Low altitude animals who deal with environmental hypoxia

One of the most interesting group of creatures who routinely are exposed to hypoxic environments are residents of tidal pools. The PO₂ of the aquatic environment depends on both the tide and the time of day (56). For example if emersion (low tide) occurs during the day, the pool is isolated from the ocean and aquatic plants undergoing photosynthesis produce oxygen. In these circumstances, the PO₂ of the tidal pool water approaches 500 Torr. However if the pool becomes isolated at night, the plants consume available oxygen and the ambient oxygen levels approach zero. During immersion (high tide) as the pool is flushed by fresh sea water, the partial pressure approaches that of sea level atmospheric i.e. 150 Torr. Thus, these animals must cope with a 500-fold variation in oxygen availability where conditions may change rapidly, with seconds, minutes or a few hours.

Many species of animals spend a significant portion of time in the aquatic environment and experience hypoxemia during diving. Diving animals exhibit a consistent pattern of response that is conserved across species: Apnea, bradycardia, peripheral vasoconstriction and hypometabolism in vasoconstricted area. These diving species can be placed into two behavioral categories 1) divers, such as the fur seal and 2) surfacers such the elephant seal (29). Divers are animals who during a 24-hour period at sea spend most of their time on the surface. They dive in bouts often repeatedly to hunt and capture prey. Surfacers on the other hand, spend most of their time at sea underwater and surface only to breathe. The northern elephant seal shows a pattern occurring over several days, of virtually continuous deep dives, of up to 600m in depth (31), although much deeper dives have been recorded.

By comparison, the Ama (sea woman) of Korea and Japan dive for approximately one hour at a time and to depths of less than 20 m for foodstuff harvested off the sea floor (21). The current human breath-hold diving record for single dive is a depth of 531 feet (162 meters) on a single breath of air.

Other examples of species exposed to hypoxia at sea level include burrow dwellers such as the mole rat. The blind mole rat (*Spalax ehrenbergi*) lives

in a complex burrow system and is continually exposed to a hypoxic hypercapnic environment (2). These animals exhibit a profound hypoxia tolerance (1) and exhibit several adaptations that may be important such as increased capillary density and increased pulmonary diffusing capacity (62). In addition, neonates from many species are hypoxia tolerant, and the ability to withstand hypoxic stress far exceeds that of adults (37).

OXYGEN SUPPLY

Ventilation

Adaptive responses to hypoxia can be considered in terms of oxygen supply and demand. The first step in oxygen supply lies with ventilation and the delivery of oxygen into the circulation. It has been noted that human high altitude climbers exhibit a brisk hypoxic ventilatory response when compared to both sedentary controls and to marathon runners (45). This may confer an advantage both in terms of elevating alveolar PO_2 , but also in leftward shifting the oxygen hemoglobin dissociation curve because of profound respiratory alkalosis (61). Although O_2 exchange is similar in humans and birds resting at sea level, the avian lung is more efficient than an alveolar lung at O_2 loading at altitude. Cross-current gas exchange in the parabronchial lungs of birds results in lower PCO_2 values and higher PO_2 values (41). Also, birds hyperventilate more than mammals at altitude, apparently because they can tolerate lower arterial PCO_2 levels. The low PCO_2 levels raise PO_2 , allowing birds to tolerate acute exposures to altitudes and hypoxic levels that would cause unacclimatized mammals to lose consciousness (41).

Another animal that exhibits a unique ventilatory strategy is the bat. Under conditions of torpor, bats can sustain very low metabolic rates, and employs oxygen conserving strategies. For example, the big brown bat (*Eptesicus fuscus*) couples its ventilation to metabolic rate. Above 30 °C this animal breathes continuously (52) but at a body temperature of 20 °C the respiratory rate is two orders of magnitude lower than at 30 °C and approaches that seen in some resting reptiles (22). In these animals blood and lung O_2 can account for only 21% of the metabolic requirements; much of the balance is likely supplied by diffusion and non-ventilatory bulk convection down the airways past an open glottis (51).

Mudskippers (e.g. *Periophthalmodon schlosseri*) are air breathing fish that inhabit tidal flats and actively emerge from the water for foraging, courtship and territorial (12). They also build burrows for reproduction and actively defend these territories. Because of the reducing environment of the tidal flat, the burrows are filled with nearly anoxic water (56). These animals

store large bubbles of air in their burrow and when forced to retreat into this structure, breath from this alternate air supply. In addition, these animals seldom ventilate their gills as long as air is available, and therefore suffer no net loss of PO_2 when exposed to severely anoxic water (26). Also many mudskippers rely on cutaneous respiration uptake and may receive up to 80% of total oxygen uptake by non-ventilatory sources (53).

Gas Exchange

The bird exhibits features of gas exchange that may explain its remarkable hypoxia tolerance. Gas exchange in the parabronchial lungs of birds is theoretically more efficient than alveolar gas exchange in alveolar lungs but this is not always observed in (41). The unique anatomy of avian lungs results in a cross-current model of gas exchange in which arterial PO_2 can exceed end-expired PO_2 and arterial PCO_2 can be less than end-expired PCO_2 . Although this behavior for CO_2 is observed commonly in birds resting or exercising at sea level, it is not observed for O_2 under similar conditions. The main reason for this is that ventilation-perfusion heterogeneity occurs in avian lungs, similar to mammalian alveolar lungs, and the impact of such heterogeneity is greater on O_2 exchange than on CO_2 exchange. This is because of differences between the dissociation curves for these gases in blood, also similar to alveolar gas exchange, although the discrepancy between the effects of a given amount of heterogeneity on O_2 vs. CO_2 is greater in cross-current gas exchange (41).

Table 1 gives ventilation-perfusion heterogeneity data obtained from inert gas studies conducted at rest and during exercise from several different species. The log standard deviation of the perfusion distribution (LogSDQ) is used as an index of ventilation perfusion matching with the greater the LogSDQ the greater the extent of ventilation-perfusion heterogeneity. It can be appreciated that in most animals, exercise results in an increasing in the LogSDQ from rest to exercise, and in many species this is either unchanged or worsened in hypoxia. In the Emu, the LogSDQ is, if anything, reduced with hypoxic exercise, and the overall increase of the ventilation relative to perfusion minimizes the effect of the heterogeneity on gas exchange (44).

Table 1. Ventilation-Perfusion heterogeneity ($\log SD_Q$) in mammals, reptiles and birds during exercise and hypoxia. Means \pm S.E.M.

	Normoxic rest	Normoxic exercise	Hypoxic rest	Hypoxic exercise
Mammals				
Horse [58]	0.33 \pm 0.01	0.39 \pm 0.02	0.30 \pm 0.01	0.37 \pm 0.02
Pig [24]	0.42 \pm 0.04	0.69 \pm 0.04		
Human [39]	0.45 \pm 0.03	0.54 \pm 0.05	0.48 \pm 0.05	0.62 \pm 0.9
Aerobic Athletes [23]	0.54 \pm 0.04	0.67 \pm 0.02		
Reptiles				
Alligator [40]	0.47 \pm 0.06			
Monitor Lizard 1b [22]	0.39 \pm 0.06	0.78 \pm 0.05		
Birds				
Geese [40]	0.56 \pm 0.30			
Emu, [44]	0.60 \pm 0.06	0.68 \pm 0.06	0.63 \pm 0.07	0.62 \pm 0.03

See text for explanation

The horse also experiences very tight matching of ventilation to perfusion and the $\log SD_Q$ of the horse during exercise, even in hypoxia, is less than most mammals at rest in normoxia (57, 58). However, this gas exchange attribute alone does not confer hypoxia tolerance and the horse experiences profound hypoxemia during sea level exercise. The mechanism for this is twofold: During galloping, there is alveolar hypoventilation and some animals may retain CO_2 above resting levels. In addition the alveolar-arterial difference for oxygen ($AaDO_2$) widens, and up to 75% of the $AaDO_2$ is due to diffusion limitation of oxygen transport. The phenomenon of exercise induced arterial hypoxemia in humans is well described in (13, 23) and those that exhibit the most marked hypoxemia are highly aerobic athletes where diffusion limitation may explain 75% of the $AaDO_2$ (42).

It is therefore clear that a large diffusing capacity for oxygen is important in maintaining oxygen transport, especially in light of declining driving pressure for oxygen caused by hypoxia and during exercise. There is a tight linear relationship ($R^2 > .95$) between \log body size and \log surface area of the blood gas tissue barriers for both birds and mammals, and for both groups they scale as a power of 0.88 of body size (34). However in the bat, this relationship is significantly altered and the respiratory surface area is similar to an animal which is 4-5 times larger (34). This is because the alveoli of the bat are relatively smaller than other mammals allowing more surface area for diffusion (33). This in part explains the large metabolic scope of these animals: the difference in oxygen uptake between torpor and flight is 1200 fold (50). In contrast, normal humans have a metabolic scope

of approximately 10 fold, reaching 20 fold only in the most highly trained athletes.

Oxygen Transport in Blood

Perhaps one of the most robust adaptations to hypoxia, seen across widely divergent species, relates to the affinity of hemoglobin for oxygen. This has been the subject of several excellent reviews (54, 59) and will be on briefly discussed here. Generally, there is a relationship between body size and P_{50} with small animals having lower affinity hemoglobin than larger animals. Smaller animals have a higher mass specific metabolic rate and may be selected to favor oxygen unloading to the tissues (54). Thus, it is important when examining these relationships to choose animals of similar body size. Although it is difficult to compare directly across species e.g. bird to man, when comparing hypoxia tolerance in a species to a similar species or subspecies that is not particularly noted for its hypoxia tolerance a remarkable pattern emerges. Figure 1 shows the P_{50} for 4 different vertebrate classes with two mammalian species. In these different animals, the oxygen hemoglobin dissociation curve of the hypoxia tolerant animal is left shifted in the hypoxia tolerant species, favoring oxygen loading. The bar headed goose has a higher affinity than the Canada goose, which flies at relatively modest elevations (43). The high altitude champion the Ruppell's Griffon has a P_{50} of 16.4 Torr, lower than any of the other animals discussed (60). The blind mole noted for its hypoxia tolerance (1) has a higher affinity hemoglobin that does it more surface dwelling relative the white rat (54). Similar relationships are found between the green sea turtle a carnivore which repeatedly dives in search of prey, compared to the more herbivorous and shallow water dwelling loggerhead turtle. The mudskipper who tolerates almost complete anoxia on tidal flats has a lower P_{50} than does the lungfish (27) another air breathing. In humans, Sherpas have a lower P_{50} than do acclimatized lowlanders, although this relationship does not hold true for Andean natives (36). Thus with few exception, hemoglobin which favors oxygen loading in the lung, is a feature of many hypoxia tolerant species, and likely represents a common theme of hypoxia tolerance. Remarkably, this trait is independent of the absolute values of P_{50} in the low altitude species.

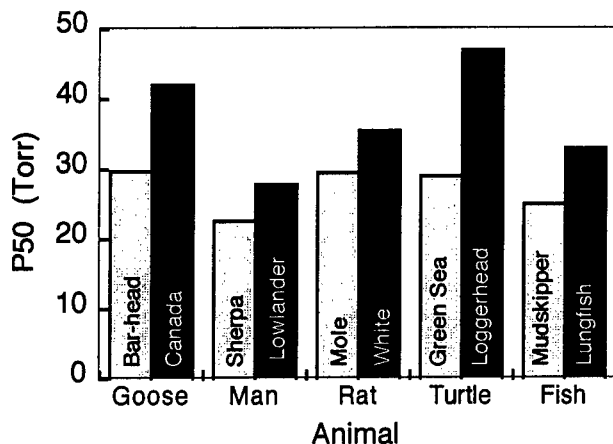


Figure 1. Legend P_{50} for hypoxia tolerant (gray) and related species (black). In all cases cited the hypoxia tolerant animal has a greater affinity hemoglobin for oxygen (see text for references).

Another theme of hypoxia tolerance is the ability to regulate distribution of blood flow. For example, in *Trachemys (Pseudemys) scripta* as for many reptiles, brief ventilatory periods are interspersed among apneas of variable duration (47). During apnea, a bradycardia develops and there is a significant increase in the pulmonary vascular resistance, which leads to a reduction in pulmonary blood flow (48). During brief ventilatory periods, the heart rate increases, the pulmonary vascular resistance decreases and blood flow to the lungs can increase as much as 5-7 fold. At least 50% of the increase in pulmonary vascular resistance lies proximal to the lung (see 7), where the bulbus cordis provides variable constriction in response to apnea (7). Circulatory adaptations to diving are well known in turtles, birds and pinnipeds (see (28)). Beside the changes in pulmonary circulation in the turtles described above they include bradycardia, peripheral vasoconstriction and blood flow redistribution. Bradycardia is a predominant feature and diving heart rates tend to be less than or equal to resting surface eupneic rates (28). Peripheral vasoconstriction during diving, was described in penguins by Scholander in 1940 (46) and is well documented in many species. More recent work has found a variable response between species that depends on the nature of the dive. Muscle blood flow is reduced and the pattern of the reduction may be to a trickle, small pulses or entire cessation (4).

Cerebral cortical blood flow may be reduced early in diving seals, when arterial blood oxygen levels are relatively high, however it is restored later in the dive when oxygen saturation is reduced. Brain stem blood flow appears to be maintained throughout (5). One remarkable feature of birds is the

ability to maintain cerebral blood flow in the face of hypocapnia. Figure 2 gives relative changes in cerebral blood flow compared to control conditions for similar levels of hypocapnia. Reduction in cerebral blood flow for human, monkeys and rats ranges from 40 to almost 60 percent. By contrast, the reduction in cerebral blood flow by birds in the face of hypocapnia is essentially zero (16). Thus, the ability to maintain cerebral blood flow appears to be a common trait among several hypoxia tolerant species.

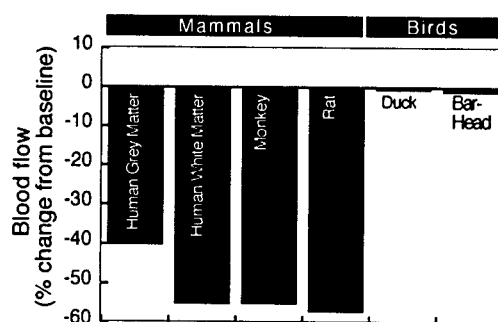


Figure 2. Changes in cerebral blood flow for three mammals and two bird species. The bird is remarkable in that it is able to maintain cerebral blood flow in the face of hypocapnia. See text for explanation and references.

Gas exchange at the tissue

A large tissue diffusing capacity for oxygen would be ideal to facilitate oxygen delivery during conditions of reduced availability. It was long thought that adaptation to hypoxia resulted in an increased capillary to fiber ratio, which might facilitate oxygen delivery to tissues. However, it is now recognized that this apparent increase in capillarity results from a decrease in fiber surface area when exposed to hypoxia (3). An exception to this is the avian response. When acclimatized to high altitude pigeons show an increased in capillary fiber number associated with an increase in tortuosity and branching, without a decrease in fiber cross-sectional area (35). When high altitude finches are compared with the same species living at low altitude the same changes hold true with the additional change of an increase in mitochondrial volume (17). This effectively increases the diffusing capacity for tissue oxygenation in these high altitude native animals.

OXYGEN DEMAND: REGULATION OF METABOLIC RATE

The ability to depress metabolic demand is another common theme of many hypoxia tolerate species. This response is well documented in reptiles, small mammals and neonates. For example, most ectotherms live in an

environment with a mosaic of temperatures. Through both behavioral (e.g. sun vs. shade) and physiological mechanisms (e.g. altering body surface area exposed for radiation or conduction) these animals are able to maintain body temperatures different from ambient. When given a range of temperatures to choose from in a thermal gradient these animals will seek a specific temperature, referred to as a preferred body temperature. When exposed to hypoxia, lizards will reduce their preferred body temperature thus lowering their oxygen demand. This is not a graded phenomenon but appears to have threshold for response at a FIO_2 of about 10% (18). Newborn mammals when faced with environmental hypoxia respond by a reduction in metabolic rate. This results in a hyperventilation as ventilation is maintained or even increased (37). The extent to which metabolism is depressed, in part depends on the extent of the ventilatory response, and those species which exhibit a marked ventilatory response, show a lesser reduction in metabolism. This effect occurs regardless of ambient temperature, although hypoxia alone will decrease thermogenesis and lowers the preferred ambient temperature (37). This is also seen in adult animals with small body mass and the magnitude of the hypoxic depression of metabolism is approximately proportional to the

mass specific $\dot{V}O_2$ of the animal. However adult humans do not appear to exhibit hypoxic hypometabolism and basal oxygen consumption at altitude is elevated about sea level mean values some 15% even several days after arrival at altitude (6).

Conservation of supply

In addition to reducing metabolic demand by hypometabolism, many species employ oxygen saving strategies during exercise. For example, it has recently been demonstrated that seals, dolphins and whales that were instrumented using small underwater video cameras spend a significant portion of their diving time, gliding (63). In seals stroking was generally seen during the first 100m or so in depth, and then the animals employed long periods of gliding up to 6 minutes in duration until a maximum depth was reached. Descent is possible because hydrostatic forces compress the air in the lungs and volume decreases without a change in mass, thus the buoyancy decreases on descent. To surface the animals exhibited stroking interspersed with gliding until shortly before or while attaining the surface. Similar behavior was observed in whales and dolphins who have vastly different body sizes and swimming mechanisms (63).

Flapping (active) light is an energetically costly means of locomotion unless considered in terms of cost per unit distance. At fast speeds, the distance covered per unit of energy is less than swimming, running or walking (32). Hovering flight is the most energetically expensive form of flight and is more than double the cost of forward flight (32). However, many

birds exploit air currents for gliding or use "flap-gliding" gaits to conserve energy (9).

Alterations in Substrate utilization

In addition to conserving available oxygen store by efficient forms of locomotion there is evidence that substrate utilization may be altered under conditions of hypoxia to favor maximal ATP generation. This has been reviewed extensively (e.g. (19, 20) and it is outside the scope of this paper to discuss it here.

COMMON THEMES AND CONCLUSION

The adaptive responses to environmental hypoxia are almost as diverse as the animals themselves. However, several traits appear to be conserved among vastly different hypoxia tolerant creatures. These are: a left shifted oxygen hemoglobin dissociation curve, the ability to lower metabolic rate in response to hypoxia, the ability to redistribute blood flow to maintain cerebral oxygenation and the ability to employ behaviors optimizing oxygen utilization. Mechanisms responsible for common and diverse features of adaptation to altitude may be revealed by future comparative studies. Evolutionary physiology offers the most promising approach, using comparative physiology to study species with appropriate phylogenetic relations, complemented by studies of genetically engineered or artificially selected animals (*cf.* chapter 9, T. Garland's contribution to this volume).

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Chapter 12

Biology of erythropoietin

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Abstract: Hypoxia induces tissue-specific gene products such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which improve the peripheral O₂ supply, and glucose transporters and glycolytic enzymes, which adapt cells to reduced O₂ availability. EPO has been the fountainhead in research on pO₂-dependent synthesis of proteins. The EPO gene enhancer (like the flanking DNA-elements of several other pO₂-controlled genes) contains a consensus sequence (CGTG) that binds the trans-acting dimeric hypoxia-inducible factor 1 (HIF-1 α / β). The α -subunit of HIF-1 is rapidly degraded by the proteasome under normoxic conditions, but it is stabilized on occurrence of hypoxia. HIF-1 DNA-binding is also increased by insulin, and by interleukin-1 and tumor necrosis factor. Thus, in some aspects there is synergy in the cellular responses to hypoxia, glucose deficiency and inflammation. In viewing clinical medicine recombinant human EPO (rHu-EPO) has become the mainstay of treatment for renal anemia. Endogenous EPO and rHu-EPO are similar except for minor differences in the pattern of their 4 carbohydrate chains. rHu-EPO is also administered to patients suffering from non-renal anemias, such as in autoimmune diseases or malignancies. The correction of anemia in patients with solid tumors is not merely considered a palliative intervention. Hypoxia promotes tumor growth. However, the benefits of the administration of rHu-EPO to tumor patients with respect to its positive effects on tumor oxygenation, tumor growth inhibition and support of chemo- and radiotherapy is still debatable ground.

Key words: hypoxia inducible factor, HIF-1, recombinant human erythropoietin, anemia, tumor oxygenation

INTRODUCTION

Hematocrit and blood hemoglobin concentration are maintained at a constant level in non-hypoxic healthy adult persons. Erythropoiesis compensates for the permanent destruction of aged red blood cells by macrophages in bone marrow, spleen and liver. About 1 % of the red cell mass is renewed each day. The basal rate of the production of red cells may increase up to 10fold following a blood loss or on exposure to high altitude. The hormone erythropoietin (EPO) is required for the elaboration of red cells. The main stimulus for EPO synthesis is tissue hypoxia. Lack of EPO results in anemia, as seen in chronic renal disease or inflammation. Anemic persons suffer from fatigue and reduced work capacity. Treatment with recombinant human EPO (rHu-EPO) has become an important medical tool to increase the quality of life of previously anemic patients.

The interrelation between EPO and tissue oxygenation is considered from several perspectives in this article, including (a) the physiologic function of EPO in red cell production, (b) the transcription factors controlling EPO synthesis, (c) disorders of EPO production, (d) structural and biological characteristics of rHu-EPO preparations and their clinical use and (e) relations between anemia, tumor oxygenation and efficiency of anti-tumor therapy.

EPO in the regulation of red cell production

Experimental studies in animals have shown that almost all circulating EPO originates from peritubular interstitial cells in the cortex of the kidneys and from parenchymal cells in the liver (45,46). In addition, some EPO mRNA has been detected in spleen, lung, testis and brain (18,44,60). The O₂ capacity of the blood is the primary determinant of the rate of EPO synthesis in kidneys and liver (33). There is an exponential increase in the concentration of circulating EPO with decreasing blood hemoglobin concentration in anemic patients without renal failure or inflammatory disease. EPO levels in plasma may reach 10,000 IU/l (international units per liter) in severe anemia, compared to a normal value of about 15 IU/l (Figure 1). In addition, EPO synthesis is stimulated when the arterial O₂ tension is lowered or when the O₂ affinity of the blood is increased. Changes in renal blood flow have little influence on EPO production (50). A few other hormones can interfere with the pO₂-dependent feedback circuit of erythropoiesis (33). For example, the higher hemoglobin and hematocrit values in male subjects compared to females are probably due to the myeloid action of androgens, which augment the effect of EPO. Thyroid hormones, epinephrine, insulin-like growth factor I and angiotensin II may also stimulate erythropoiesis.

EPO stimulates the growth of erythrocytic progenitors (burst-forming and colony-forming units erythroid: BFU-E and CFU-E) which are progenies of pluripotent stem cells in the hemopoietic organs. The functional human EPO receptor (EPOR) is a 484-amino acid glycoprotein and member of the class I cytokine receptor superfamily. The transmembrane EPOR molecules form homodimers. On binding of EPO to the EPOR a conformational change of the intracellular receptor-domains initiate the activation of 2 receptor-associated tyrosine kinases (JAK2, Janus kinase 2). Once initiated, the two JAK2 molecules activate themselves by reciprocal tyrosine phosphorylation. Activated JAK2 phosphorylate then the EPOR and several distinct intracellular signaling molecules. The most important "downstream" molecule transmitting EPO effects is STAT5 (Signal Transducer and Activator of Transcription). STATs are latent cytoplasmic transcription factors which, as soon as they become activated (via action of JAKs), dimerize and translocate into the nucleus where they bind to specific DNA sequences and allow the respective genes to be transcribed (39). Suppression of apoptosis, the programmed cell death, is the primary mechanism by which EPO maintains erythropoiesis (40). When the concentration of the hormone rises in blood an increasing number of progenitor cells escape from apoptosis. The subsequent molecular events that lead to proliferation and differentiation of erythrocytic cells are only partially understood. Three to 4 days after an acute increase in plasma EPO reticulocytosis becomes detectable.

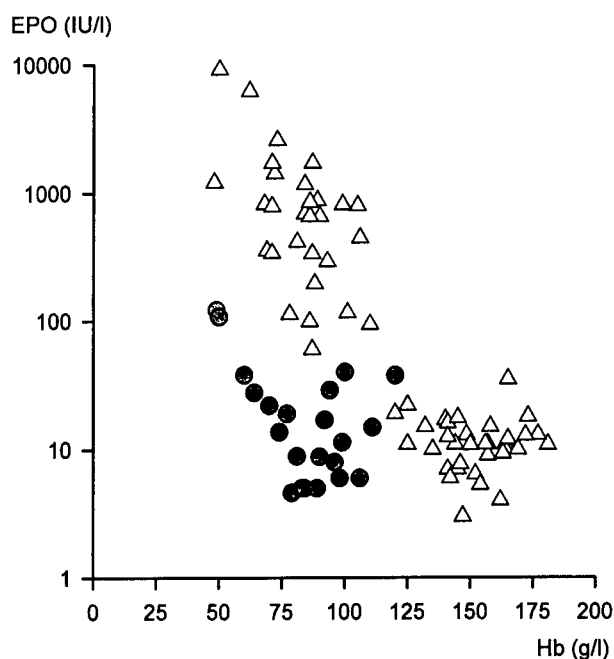


Figure 1. Serum erythropoietin (EPO) concentrations in dependence of blood hemoglobin (Hb) levels in subjects with normal kidney function ($n = 63$, triangles) and in patients with chronic renal failure ($n = 22$, circles). EPO was assayed by enzyme-linked immunoassay (36).

Transcription factors controlling EPO synthesis

Although the molecular mechanisms of O_2 sensing have been in the focus of extensive research the initial steps are still incompletely understood (51). Possibly, heme proteins function as O_2 sensor (1,9,17). Most of the current knowledge has been derived from in vitro studies utilizing EPO producing human hepatoma cell cultures and, in detail, may not hold true for the O_2 sensing mechanism in control of the renal EPO synthesis. EPO gene expression in hepatocytes occurs in a graded fashion. In contrast, EPO gene expression in the kidney follows an all-or-none rule, i.e. the increase in EPO mRNA with increasing severity of hypoxia is due to the recruitment of additional cells, all of which are maximally active (41). Some investigators have assigned a positive role for mitochondrial cytochrome-derived reactive O_2 species in the hypoxic induction of EPO synthesis (13). However, because cyanide does not interfere with pO_2 -dependent in vitro synthesis of EPO ((37) and own observations), the role of mitochondria in O_2 sensing needs further elucidation. In addition, evidence suggests that b-type cytochromes produce H_2O_2 and other reactive O_2 species inhibiting EPO gene expression at high pO_2 (16,25). The rate of the production of H_2O_2 decreases in human hepatoma cells on lowering pO_2 (19). It has been proposed that H_2O_2 inhibits EPO gene expression via activation of GATA-2 (31,59). GATA-2 is a member of the GATA family of transcription factors. Its mode of action on the EPO gene is somewhat unique. Binding to the GATA-motif, located in the -30 region relative to the transcriptional initiation site of the EPO promoter, represses transcription of the gene (31,65). Thus, GATA-2 is the primary candidate causing the normoxic repression of the EPO gene.

Some other transcription factors are of relevance for the strict regulation of the EPO gene in vivo (Figure 2). Evidence suggests that HNF-4 (hepatocyte nuclear factor 4) is necessary for full hypoxic inducibility whereas COUP-TF1 (chicken ovalbumin upstream promoter-transcription factor 1), which binds to the same target sequence in the EPO enhancer, acts antagonistic to HNF-4 (9). The role of NF κ B, one of the most important signal molecules involved in inflammatory processes, is still poorly understood with respect to the control of EPO gene transcription. However, the cytokines TNF- α (tumor necrosis factor α) and IL-1 β (interleukin 1 β) downregulate EPO production (20,21,28). Blockade of NF κ B activity abolishes the cytokine-induced inhibition of EPO gene expression (own unpublished observation). Lack of canonical CAAT- and TATA-boxes and a high GC-content of the EPO promoter are indicative for a housekeeping function.

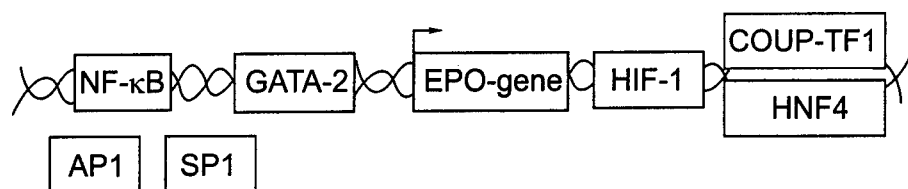


Figure 2. Scheme showing selected regulatory elements (open boxes) of the promoter and enhancer regions of the EPO gene.

A regulatory trans-acting protein of major importance is the so-called hypoxia inducible factor 1 (HIF-1), which binds to the hypoxia-responsive element (HRE) in the 3'-flanking enhancer of the EPO gene. HIF-1 is a ubiquitously and constitutively expressed transcription factor. It is composed of 2 different subunits, the 120 kDa HIF-1 α and the 91-94 kDa HIF-1 β (68). The HIF-1 α subunit is unstable at high pO_2 , while the β subunit is permanently present in nuclei (Figure 3). HIF-1 α possesses an oxygen-dependent degradation domain that targets the protein for immediate ubiquitination and proteasomal lysis under normoxic conditions (55). This process involves the von-Hippel-Lindau (VHL) tumor suppressor protein (47). The additional finding that cobaltous ions and iron chelators such as desferrioxamine prevent the formation of the VHL complex (47) may provide an explanation for the established stimulation of EPO synthesis by these compounds (33). Furthermore, the function of another tumor suppressor gene product, PTEN (phosphatase and tensin homolog deleted on chromosome ten), is critical for normoxic degradation of HIF-1 α (74,75). While HIF-1 α is almost undetectable in normoxic cells, the HIF-1 α/β complex can be demonstrated in nuclear extracts within minutes after exposure of cells to hypoxia (Figure 4). HIF-1 α/β binds to the consensus DNA binding sequence A/(G)CGTG present in the HREs of the promoter or enhancer of many pO_2 -controlled genes (Figure 5). Consequently, HIF-1 α/β regulates the synthesis of proteins that protect the organism against hypoxia, such as EPO, vascular endothelial growth factor and distinct glycolytic enzymes (9,56,69).

Originally, it was assumed that HIF-1 α was induced by hypoxia alone. However, recent studies have identified a number of hormones and cytokines capable of inducing HIF-1 α/β DNA-binding. The observation that both insulin and insulin-like growth factors (IGF) 1 and 2 activate HIF-1 α is relevant with respect to the induction of membrane-bound glucose transporters and glycolytic enzymes (22,72). The activation of HIF-1 by interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF- α), epidermal growth factor and fibroblast growth factor 2 indicates an overlap in the cellular responses to inflammation and hypoxia (28,64,72). The capability to

increase HIF-1 α / β DNA-binding does not necessarily mean that the stimulus initiates EPO gene expression (Figure 6). The proinflammatory cytokines IL-1 and TNF- α lower EPO production (20,21,28,35), while they upregulate other HIF-1 dependent genes such as those encoding VEGF or inducible

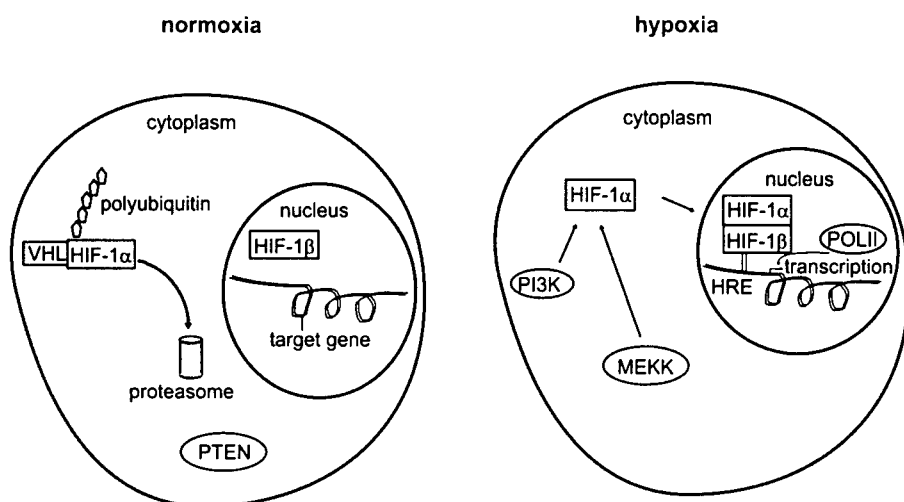


Figure 3. Model showing the different metabolic pathways of HIF-1 α in normoxic versus hypoxic cells. In normoxia HIF-1 α is rapidly polyubiquitinated and subsequently degraded via the proteasome. Degradation occurs only in the presence of functional products of the VHL- (von Hippel Lindau) and the PTEN- (phosphatase and tensin homolog deleted on chromosome ten) genes. In hypoxia the HIF-1 α subunit is stabilized and enters the nucleus. This process seems to require the action of the PI3K- (phosphatidylinositol 3-kinase) and the MEKK- (MAP kinase kinase) pathways. HIF-1 α heterodimerizes with HIF-1 β in the nucleus and the dimer binds to the HREs (hypoxia-responsive elements) of HIF-1 target genes. In cooperation with other transcription factors and common transcriptional coactivators (X) RNA-polymerase II (POL II) is directed to the transcriptional start site of the respective gene.

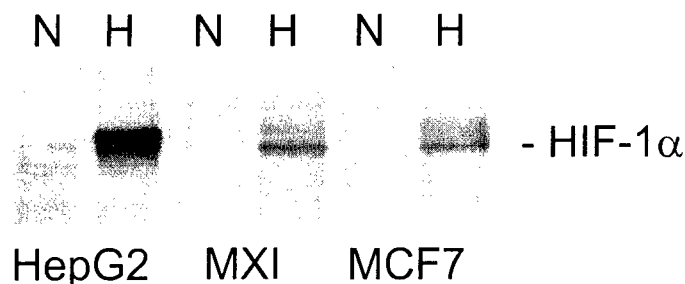


Figure 4. HIF-1 α Western blots with nuclear extracts from normoxic (20% O₂, N) and hypoxic (3% O₂, H) human cell cultures (HepG2 : hepatoma; MX1 and MCF7 : mammary cancer). The incubation period was 4 h.

NO-synthase. Figure 7 illustrates the complex interplay of the various transcription factors during physiological and pathophysiological conditions. Despite the increase of HIF-1 DNA binding activity EPO transcription is reduced during inflammation due to lowered HNF-4 (see above) and elevated levels of the negatively regulating transcription factors GATA-2 and NF κ B.

Hypoxia-induced synthesis of EPO is not restricted to the kidneys and the liver. Of special interest are observations indicating that the expression of the EPO gene as well as of the EPO receptor gene is also stimulated by hypoxia in brain. Neuronal EPO is probably not involved in the control of erythropoiesis. Instead, EPO in brain is thought to act as an anti-apoptotic tissue hormone. The molecular mechanisms of the neuroprotective effect of locally produced EPO are not fully understood. It has been proposed that EPO inhibits the formation of reactive O₂ species and N-methyl-D-aspartate receptor-mediated cytotoxicity (53,54).

Normal and abnormal levels of serum EPO

The concentration of serum immunoreactive EPO is 6 - 32 IU/l in non-anemic humans. Overproduction of EPO may occur as a physiological response to hypoxia, e.g. at high altitude, or arise autonomously as a paraneoplastic syndrome (Table 1). Excessive EPO production is probably the major pathogenetic factor in the development of chronic mountain sickness. If hematocrit exceeds 0.50, blood viscosity and, hence, cardiac afterload increases significantly. In microvessels blood stasis may occur. Studies in mice transgenic for the EPO gene, resulting in hematocrit values of about 0.80, have shown that severe chronic erythrocytosis leads to intracellular edema of cardiomyocytes, cardiac dysfunction, impaired exercise performance and reduced life expectancy (67). The fact that arterial blood pressure is not generally elevated in such animals has been explained by an adaptive increase in NO-synthase activity which allows for a

compensatory vasodilation (52). The main risks of erythrocytosis with hematocrits > 0.55 in humans include heart failure, myocardial infarction, seizures, peripheral thromboembolic events and pulmonary embolism. In patients suffering from secondary erythrocytosis phlebotomy treatment to hematocrit 0.52-0.50 may be beneficial to prevent hemodynamic and rheological complications. However, repeated phlebotomies lead to iron deficiency in the long term. Therefore, the development of specific erythropoiesis-inhibiting drugs should be an attractive pharmacological task.

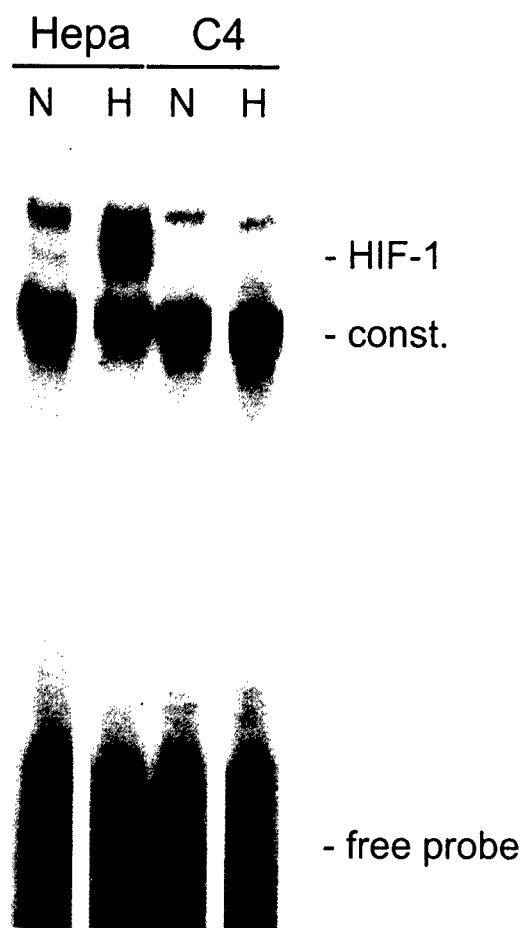


Figure 5. Scheme illustrating that the expression of hypoxia-inducible genes may be reduced (-) or stimulated (+) by IL-1 and TNF- α at increased HIF-1 α/β DNA-binding. Proposedly, the cytokine-inducible transcription factors GATA, NF κ B and AP-1 (activator protein 1) are responsible for this diversity.

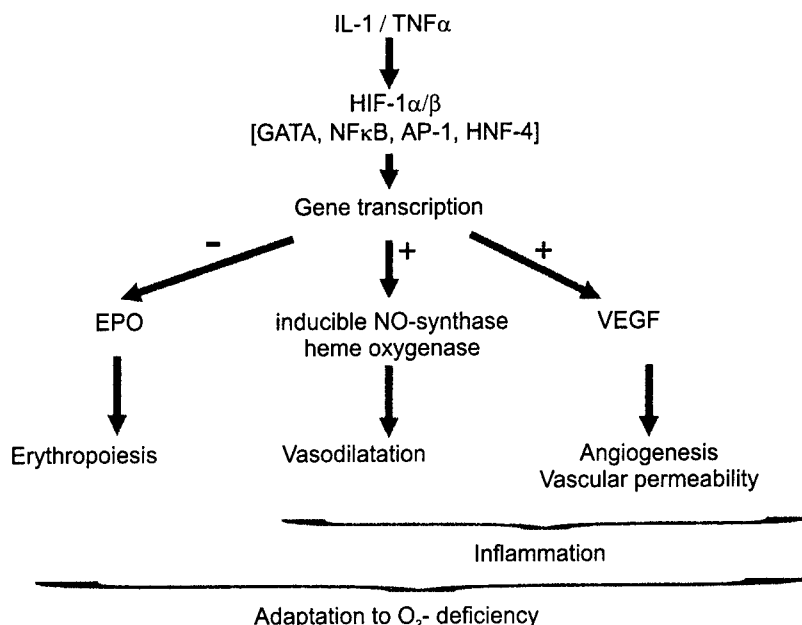


Figure 6. Concept of the control of EPO gene expression by transcription factors in normoxia, hypoxia and inflammation.

EPO deficiency results in reduced red cell formation and anemia. The clinical symptoms may vary depending on the degree of anemia, and the age and co-morbidity of the afflicted persons (Figure 8). Diseases often associated with an absolute or relative lack of EPO are noted in Table 1. Insufficient EPO production is the primary cause of the anemia in chronic renal failure (2). Apart from a possible destruction of the EPO-producing cells, the underlying pathogenetic mechanisms include inhibition of EPO synthesis due to metabolic acidosis, uremia toxins and proinflammatory cytokines. A relative lack of EPO contributes to the development of anemia in patients suffering from chronic inflammation, malignancy or AIDS (34,48). The production of EPO is not only lowered by IL-1 and TNF α (34), but also by chemotherapeutic and immunosuppressive drugs (29,71). In clinical practice the diagnosis of an "inadequate EPO response to anemia" is difficult to be made, because serum EPO levels can only be evaluated in relation to the degree of anemia (11). Thus, the definition of a relative deficiency of EPO relies on the documentation of a lowered ratio between the serum EPO level and the blood hemoglobin concentration or hematocrit (6). A widely used method is to calculate the observed/predicted log [EPO] ratio (O/P ratio), with the predicted level being estimated from a reference group of anemic patients without renal disease, infection, inflammation or malignancy. Such reference types of anemias may be iron deficiency or hemolysis.

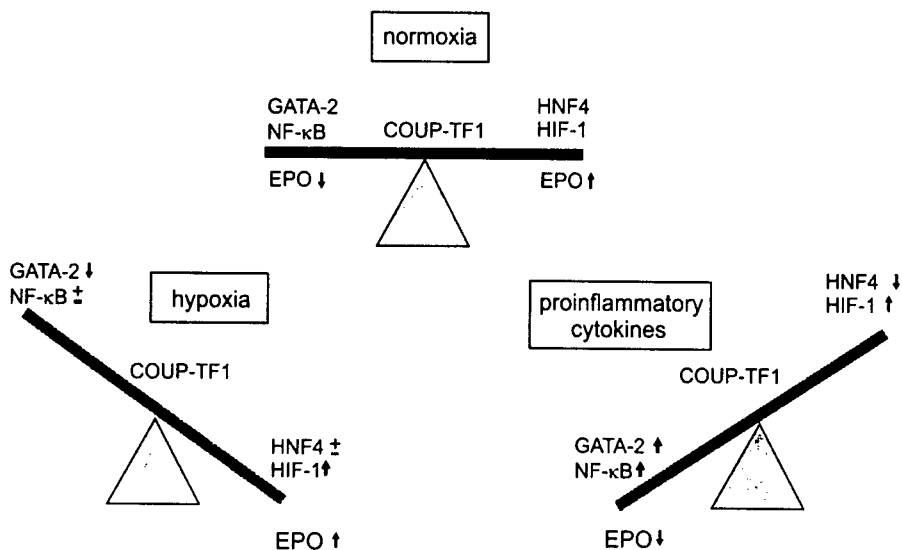


Figure 7. Pathophysiological organic and psychosomatic consequences of anemias.

Table 1. Pathophysiology of EPO production

A. Diseases often associated with EPO overproduction and secondary erythrocytosis

Chronic mountain sickness
 Chronic respiratory disease (unless complicated by inflammation or severe acidosis)
 Cyanotic heart disease
 Hemoglobinopathy with increased O₂ affinity
 Renal cysts
 Paraneoplasms producing EPO

B. Diseases often associated with EPO deficiency and anemia

Chronic renal failure
 Acute or chronic infection (including AIDS)
 Autoimmune diseases (rheumatoid arthritis, inflammatory bowel disease)
 Malignancies (solid tumors, distinct types of leukemia)
 Severe trauma (including thermal injury)

For references, see (33)

Recombinant DNA-derived EPO

Cultures of Chinese hamster ovary (CHO) cells are commonly used for the manufacture of rHu-EPO for therapy. Human urinary EPO and rHu-EPO are identical with regard to their amino acid sequence and glycosylation

sites. The peptide core of the molecules consists of 165 amino acids. The molecular mass of the glycoprotein entities is 30 kDa. The carbohydrate portion (40%) is essential for molecular stability and full in vivo biological activity. There are 3 tetraantennary N-linked (Asp 24, 38 and 83) and 1 small O-linked (Ser 126) acidic oligosaccharide side chains. The molecules form bundles of 4 α -helices which are folded into a compact globular structure. Due to differences in glycosylation, the specific in vivo biological activity of purified human urinary EPO (70,000 IU/mg peptide) is lower than that of the purified recombinant product (about 200,000 IU/mg peptide).

The biological half-life of rHu-EPO is about 4-8 h after intravenous administration. The main site of metabolism of EPO has not yet been identified. A minor portion is cleared by the kidneys. In addition, following desialylation EPO can be taken up via galactose receptors by hepatocytes, but this may not be a physiological route of degradation of the growth factor. Recently, a hyperglycosylated recombinant analogue of rHu-EPO called "novel erythropoiesis stimulating protein" (NESP) with increased biological stability has been produced for therapeutic purposes. NESP contains 2 additional N-linked oligosaccharide chains in association with 5 extraneous amino acids introduced through site-directed mutagenesis. The carbohydrate content of NESP amounts to 52% and its molecular mass to 38.5 kDa. The half-life of NESP is about 3fold longer than that of rHu-EPO in circulation (43). Therefore, it is hoped that the novel drug needs to be applied less frequently. Still, larger clinical studies will have to be carried out to prove that the rate of antibody formation towards NESP is as low in the patients as that towards rHu-EPO (1 : 200 000).

RHu-EPO therapy in renal failure

RHu-EPO therapy is effective against severe anemia in almost all patients on regular hemodialysis or continuous ambulatory peritoneal dialysis (CAPD). In addition, predialysis patients benefit from the drug (2,63). RHu-EPO produces a dose-dependent increase in hematocrit and blood hemoglobin concentration, and it abolishes the need for red cell transfusions with its risks of incompatibility reactions, infections and iron overload. In previously anemic patients, rHu-EPO therapy reverses the hyperdynamic cardiac state and restores the impaired brain function.

The complications responsible for the rare cases of rHu-EPO resistance include iron deficiency and inflammatory or infectious disease. Reduced iron availability is indicated by a serum ferritin concentration < 100 μ g/l, a transferrin saturation < 20 %, and a proportion of hypochromic red cells > 10 % (30). Resistance to rHu-EPO on infection and inflammation is mainly caused by the action of interferon γ (IFN- γ) and TNF α (3). Measurements of

C-reactive protein and baseline fibrinogen concentrations in serum may provide early recognition of the probability of response to rHu-EPO (6).

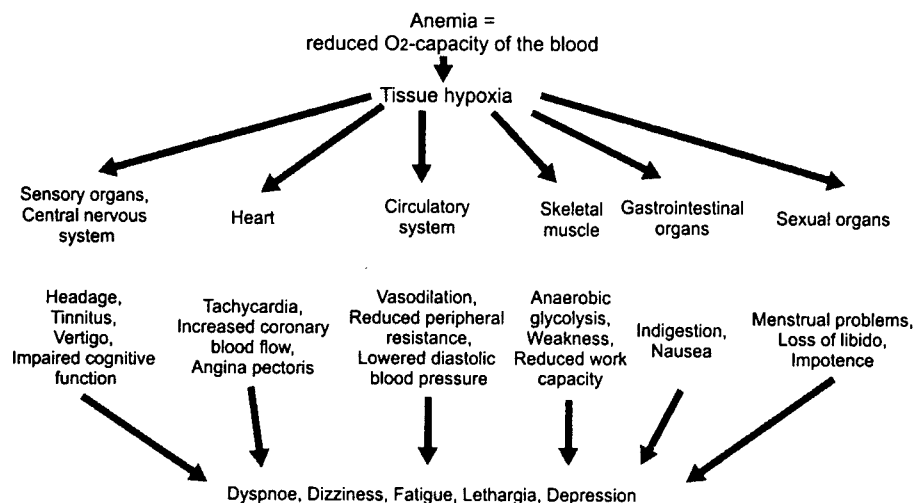


Figure 8. Pathophysiological organic and psychosomatic consequences of anemias.

The target hematocrit value is commonly set at 0.33-0.36 (hemoglobin 110-120 g/l) in renal patients under rHu-EPO therapy. The question has been raised recently as to whether a further increase in quality of life and exercise capacity could be achieved if hematocrits were increased above 0.36. However, in a prospective long-term multicenter study the one-year and two-year mortality rates were by 7% greater in the high-hematocrit (0.42) than in the low-hematocrit (0.30) group, and the study was halted after 29 months (7). Further studies are required to resolve this problem in different subgroups of patients with renal failure based on their cardiac status. Actually, the most serious side effect of rHu-EPO therapy in uremic patients is the development or worsening of arterial hypertension. The increase in blood pressure is not simply a function of hematocrit. Hemodialysis patients with a positive family history of hypertension are more susceptible to develop hypertension during rHu-EPO therapy than those with a negative family history (32). Patients with non-renal anemia do not develop hypertension on rHu-EPO therapy.

RHu-EPO therapy in non-renal anemias and for blood conservation

In some countries, rHu-EPO has been approved for treatment of the anemias associated with autoimmune diseases, AIDS, cancer, bone marrow transplantation and myelodysplastic syndromes. In contrast with the high response rate in renal anemia, rHu-EPO resistance (hemoglobin increase < 10 g/l in 4 weeks) is often seen in this diverse population of patients (4). In addition, the rHu-EPO doses ($3 \times \geq 150$ IU/kg and week) required for correction of anemia are relatively high. Attempts have been undertaken to determine predictors of the response to rHu-EPO in non-renal applications (6,12). The combination of low base-line endogenous serum EPO concentrations, a low observed/predicted (O/P) ratio of serum log EPO values, and serum ferritin < 400 μ g/l seems to provide some evidence for a positive response. Specific implications of anemia and rHu-EPO therapy for tumor progression will be discussed below.

In the surgical setting, rHu-EPO may be administered preoperatively in order to stimulate erythropoiesis in aggressive phlebotomy programs for autologous re-donation or to correct a pre-existing anemia, and postoperatively to accelerate the recovery of red blood cell mass. Sufficient autologous blood donation can prevent patients from allogeneic red blood cell transfusion (24). The beneficial effect of rHu-EPO therapy has been demonstrated in placebo-controlled, multicentre trials of patients undergoing elective hip replacement (10) or open-heart surgery (57). Maximum benefit of rHu-EPO therapy can be achieved if it is combined with iron supplementation and acute normovolemic hemodilution (8).

Anemia, tumor oxygenation and anti-tumor therapy

Anemia is a frequent complication of malignant diseases. The pathogenesis of this anemia is multifunctional. Direct effects of the tumor (bone marrow invasion, hemolysis, bleeding), inflammatory reactions ("anemia of chronic disease") and anti-tumor therapy (chemotherapy, radiation, surgery) may be involved. Although red cell survival may be shortened, the anemia is primarily of the hypoproliferative type. The concentration of circulating iron is often low due to the reduced iron mobilization from the storage sites. Cytokines such as IL-1, TNF α and interferons also inhibit the proliferation of erythrocytic progenitors directly (48). In addition, the concentration of serum EPO is relatively low in many adult cancer patients, when related to the blood hemoglobin concentration (49).

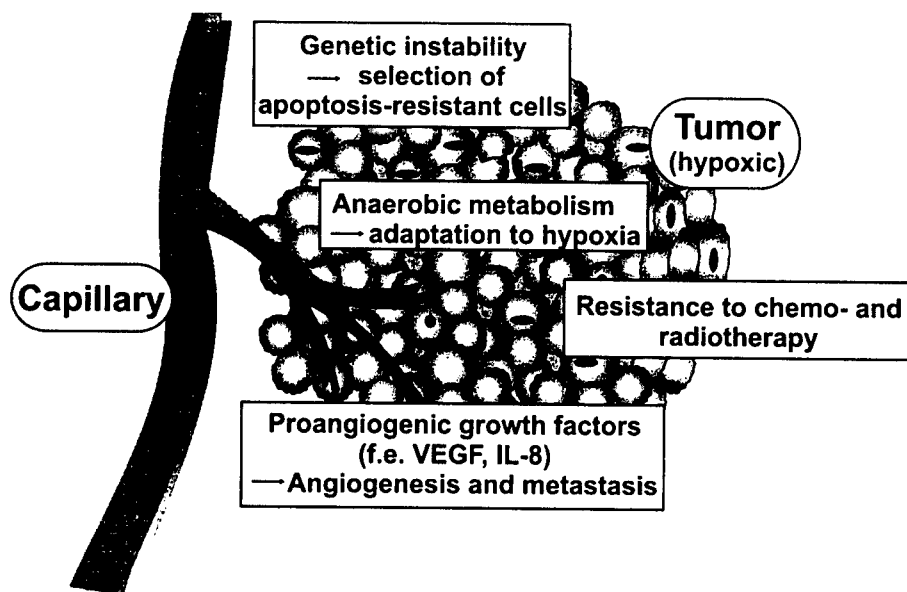


Figure 9. Mechanisms involved in hypoxia-induced malignant progression of tumors. Genetic alterations favor selective survival of cells with defects in apoptosis, overexpression of glycolytic enzymes enables anaerobic metabolism and induction of angiogenesis by VEGF and other endothelial growth factors promotes tumor expansion and metastases. In addition, hypoxia causes resistance to radiation and chemotherapy.

Several randomized and nonrandomized studies have been carried out to evaluate the efficacy of rHu-EPO treatment of the anemia associated with malignancy and chemotherapy in patients with solid tumors and primary hematological disorders. The overall response rates have ranged from 40% to 85%. A significant increase in hemoglobin levels (≥ 10 g/l) and reticulocytes ($\geq 40 \times 10^9/l$) after 4 weeks of rHu-EPO treatment is considered a reliable indicator of response. However, 10% to 30% of the patients still have to be transfused despite rHu-EPO therapy (5). An important issue yet to be clarified is the impact of the blood hemoglobin concentration on tumor growth and on outcome of anti-tumor therapy. Solid tumors are characterized by regions where the cells are hypoxic due to the long O_2 diffusion distance between the incomplete vasculature and the mass of O_2 -consuming cells (58,61). Studies in animal models have shown that the intratumoral pO_2 increases when the O_2 capacity of the blood is enhanced by hemoglobin infusion (61) or rHu-EPO treatment (38). The finding that tumor oxygenation is influenced by the O_2 capacity of the blood is important, because hypoxia induces the accumulation of a number of proteins which control tumor growth (Figure 9). Among these proteins are HIF-1 α , the vascular endothelial growth factor (VEGF) and the p53 protein

(58). VEGF is the primary growth factor for vascular endothelium, thereby promoting tumor angiogenesis and metastases (23). Regarding p53, which is a key promoter of growth arrest and apoptosis, hypoxia provides a selective pressure in tumors for expansion of variants that are devoid of apoptotic potential (26). One of the key questions to be answered is whether the expression of normally pO_2 -dependent proteins such as HIF-1 α (73) and VEGF (70) are still regulated by the redox-state of the cell. Alternatively, genetic alterations could be associated with an uncontrolled overproduction thus driving the rate of the expression of relevant genes into tumor progression. Another point of interest is the relationship between the degree of hypoxia and the therapeutic responsiveness of tumors. DNA damage by sparsely ionizing radiation (γ -radiation and X-ray) is caused by reactive O_2 species. Therefore, radiation is up to 3-fold more effective in normoxic tissue ($pO_2 > 15$ mm Hg) than in hypoxic tissue. This effect is given by the so-called "oxygen enhancement ratio", OER. Likewise, the cytotoxicity of a number of chemotherapeutics (f. e. carboplatin, cyclophosphamide, actinomycin D, 5-fluorouracil) is greater in normoxic than in hypoxic cells (62). The differences are related directly to the reduced formation of reactive O_2 species and indirectly to the slowed cell cycling in hypoxia (27). Most of the studies on the relationship between hemoglobin concentration and radiation efficiency have revealed a poorer tumor control in anemic compared to nonanemic patients. It has been shown that rHu-EPO is safe and effective in raising hemoglobin levels of anemic patients with cancers of the head, neck or thorax (42), uterine cervix (15), and lung, breast or prostate (66) during radiotherapy with or without concurrent chemotherapy. Antianemic therapy is important, not only for the well-being of tumor patients, but also for improving the outcome of treatment (14).

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Chapter 13

Lessons to better understanding of hypoxia sensing *Acquired and congenital mutations resulting in polycythemia*

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Abstract: Adaptation of the organism to hypoxia has profound effect on multiple tissues including regulation of erythropoiesis, vasculogenesis, a proper regulation of embryogenesis as well as other functions. The elucidation of those congenital or acquired mutations giving rise to disease states affecting physiological systems devoted to oxygen homeostasis provides not only a practical diagnostic and potential therapeutic target, but also allows to identify the essential, non-redundant physiological pathways that may be hitherto unknown. The erythropoietin gene was the first gene expression found to be upregulated by hypoxia; the mechanism of this regulation lead to our current understanding of hypoxia sensing. Thus it is appropriate that the disorders resulting from augmented erythropoiesis are subject of this review.

Key words: erythropoiesis; erythropoietin; erythropoietin receptor; mutations in polycythemia; hypoxia-inducible factor 1; Chuvash polycythemia

INTRODUCTION: The Regulation Of Erythropoiesis And Its Relevance To Polycythemic Disorders

Polycythemia is literally translated as “many cells in the blood”. Only erythrocytosis (an alternative term for these disorders) produces polycythemia since leukocytes and platelets are present in blood in far smaller proportions. Polycythemia may be due to increased proliferation or decreased apoptosis of erythroid progenitors, or to delayed erythroid differentiation with an increased number of progenitor cell divisions.

Prolonged red cell survival, another theoretical cause of polycythemia, has not yet been described and with intact regulatory mechanisms polycythemia would be unlikely to occur. Primary polycythemia results from abnormalities expressed in hematopoietic progenitors. In contrast, circulating factors cause secondary polycythemia (69). Both primary and secondary polycythemia can be acquired or congenital (69).

Erythropoiesis is the physiological process of the production and renewal of the red blood cell mass. This process is influenced by a number of hormones, receptors and transcription factors (reviewed in Refs. 23, 47, 60). The principal hormone that regulates erythropoiesis is erythropoietin (EPO). In adults, the kidney is the main source of EPO. After erythroid commitment, erythroid progenitors express their own EPO (88). *In vitro* studies have shown that variable levels of EPO are required at various stages of erythroid maturation (47). Instead of EPO, multipotent myeloid progenitors and primitive erythroid progenitors, i.e. early burst-forming units-erythroid (BFU-E), require stem cell factor (SCF), interleukin 3 (IL-3), granulocyte/macrophage-colony stimulating factor (GM-CSF) and/or thrombopoietin (TPO) for growth (47).

EPO, O₂-sensing, and hypoxia-inducible factor

Regulation of oxygen homeostasis is critical to survival. In response to anemia or hypoxia compensatory mechanisms occur. Under normal conditions, EPO production is mediated either by reduced red blood cell mass (anemia) or decreased O₂ saturation of red cell hemoglobin, i.e. hypoxemia (reviewed in Ref. 47). Hypoxic stimulation results in increased production of hypoxia-inducible factor (HIF-1), which is the major factor for transcriptional activation of the *EPO* gene (80). HIF-1 is also found in cells that do not express EPO, thus HIF-1 is part of a widespread O₂-sensing mechanism providing transcriptional regulation of genes for vascular endothelial growth factor (*VEGF*), glycolytic enzymes and other genes (78, 79, 101). The identity of the O₂ sensor and the mechanism by which it regulates HIF-1 are unknown at the present time. Overall, HIF-1 is a physiological regulator of genes that promote cell survival under ischemia and are expressed in response to decreased cellular O₂ tension (8). HIF-1 regulates vasculogenesis, is required for proper embryonic development, elevates glucose uptake by cells, augments production of glycolytic enzymes and also plays an important role in cancerogenesis (9, 33, 72). Thus, polycythemia may be only one of many phenotypic manifestations of a congenital or acquired augmentation of the HIF-1 pathway (77).

HIF-1 is composed of two subunits, HIF-1 α and HIF-1 β [aryl hydrocarbon nuclear translocator (ARTN)] that form a heterodimer (97); only HIF-1 α is regulated by hypoxia. HIF-1 α mRNA and protein levels are

induced by hypoxia and HIF-1 α protein decays rapidly with return to normoxia; the posttranslational regulation of HIF-1 α protein accounts for majority of hypoxic regulation of this gene (80). Normoxia-induced ubiquitin-mediated degradation of the HIF-1 α protein is the major regulator of HIF-1 α level (91). Von Hippel Lindau (VHL) syndrome is a genetic abnormality of this post-translational control. The mutated VHL protein targets HIF for oxygen-dependent proteolysis (12, 53), mediated by a complex mechanism that involves an interaction of β subunit of VHL protein with other proteins (59, 92). Von Hippel Lindau syndrome is characterized by a high propensity for development of renal tumors. They occur by somatic mutations leading to a loss of heterozygosity, however, polycythemia is not an invariable part of this disease. Hemangioblastomas of the central nervous system have long been associated with secondary polycythemia; some of these tumors are associated with elevated EPO and are sometimes seen with VHL disease.

Erythropoietin receptor

The interaction of EPO with the EPO receptor (EPOR) leads to homodimerization and signal transduction that results in a) stimulation of mitogenicity of erythroid progenitor cells, b) erythroid differentiation by induction of erythroid-specific expression of proteins such as globins, glycophorins, spectrin and ankyrin, and c) prevention of apoptosis of erythroid progenitors (reviewed in Ref. 23). The cytoplasmic portion of EPOR contains a positive growth-regulatory domain that interacts with Janus tyrosine kinase-2 (JAK2) (102). Immediately after EPO binding, JAK2 phosphorylates itself, the EPOR, and other proteins such as STAT-5, thus initiating a cascade of erythroid-specific signaling (19). This JAK2/STAT-5 signaling plays a nonredundant, essential role in EPO/EPOR-mediated regulation of erythropoiesis (62, 84). The physiological consequences of the EPO/EPOR deficiency studied in animal model are striking. When we deleted the entire *EPOR* gene in mouse embryonic stem (ES) cells and generated mice homozygous for the *EPOR* deletion (*EPOR*^{-/-}), the embryos died around day 13 of gestation due to a severe anemia (Figure 1 A and B). Cells from disaggregated fetal livers of embryonic day 12 (E12) embryos were plated for hematopoietic colony assays in cultures containing high (non-physiological) concentrations of murine TPO and murine SCF. There were low numbers of non-hemoglobinized erythroid colonies derived from BFU-E (*EPOR*^{-/-}) progenitors induced to terminal differentiation (Figure 1 C). These data suggested that disruption of EPO/EPOR signaling lead to a block of terminal differentiation of committed erythroid progenitors in fetal livers. In order to determine more precisely whether EPO/EPOR interaction plays a role in erythroid lineage commitment, we created ES cell line homozygous for *EPOR* deletion (*EPOR*^{-/-}) and differentiated these cells *in vitro*. In cultures containing TPO and SCF, the *EPOR*^{-/-} and wild type (*EPOR*^{+/+}) cell lines gave rise to the same number of erythroid colonies, however, unlike *EPOR*^{+/+}, the *EPOR*^{-/-}

colonies were only partially hemoglobinized (Figure 2 A and B. For color figures please refer to http://www.hypoxia.net/book_2001). Interestingly, both $EPOR^{+/+}$ as well as $EPOR^{-/-}$ colonies expressed EPO. This observation lead us to hypothesize that EPO operating through autocrine mechanism plays a role in terminal hemoglobinization of erythroid cells. Our results and similar data published recently by others (75) suggested possible implication of erythroid EPO in adaptation to local hypoxic stress; endogenous EPO may support complete erythroid differentiation.

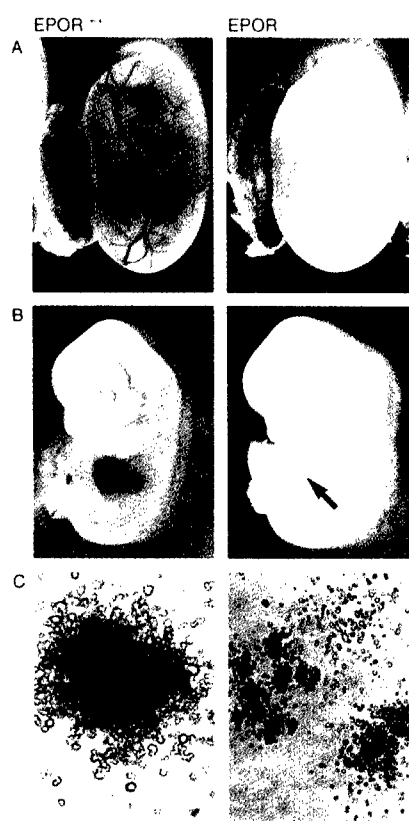


Figure 1. Analysis of erythropoiesis in $EPOR^{-/-}$ and wild-type embryos. (A) E12 embryos inside the yolk sac and (B) after removal of the yolk sac, showing severe anemia - lack of circulating red blood cells - in the mutant. The liver size of the mutant embryos was dramatically reduced (arrow) but contained committed erythroid progenitors. *In vitro* cultures (α -methylcellulose with serum and pokeweed mitogen-stimulated murine spleen cell conditioned medium, recombinant murine SCF and recombinant murine TPO) allowed a partial rescue of terminal differentiation of the mutant BFU-E progenitors (C).

The C-terminal cytoplasmic portion of EPOR also possesses a negative growth-regulatory domain (20). Hematopoietic cell phosphatase (HCP, also

known as SHP1) interacts with this portion of the EPOR and down-modulates signal transduction (37). Recruited by EPOR tyrosine Y429, HCP attaches to the cytoplasmic EPOR domain and dephosphorylates JAK2. Inactivation of the HCP binding site was shown to lead to prolonged phosphorylation of JAK2/STAT-5 (5, 37). Another negative regulator of erythropoiesis CIS3 (also known as SOCS3) also binds to the cytoplasmic portion of the EPOR. By binding to EPOR Y401, CIS3 suppresses EPO-dependent JAK2/STAT-5 signaling (51, 74). Thus, deletion of the C-terminal cytoplasmic portion of EPOR in a truncated EPOR abolishes negative regulatory elements and results in an increased proliferation of erythroid progenitor cells.

Attempts to link a number of disorders characterized by dysregulation of erythropoiesis with mutations in *EPO* and *EPOR* genes have demonstrated that only a small proportion of cases of primary familial congenital polycythemia (PFCP) are caused by *EPOR* mutations (30, 45). *EPOR* mutations are only rarely found in erythroleukemia (49). Polycythemia vera (PV) and Diamond-Blackfan anemia are not caused by mutations in *EPO/EPOR* (reviewed in Ref. 30).

Insulin-like growth factor-I (IGF-I), angiotensin II and angiotensin II type I receptor and erythropoiesis

Although *in vitro* studies of erythropoiesis have provided crucial information about the hierarchy of regulation of erythropoiesis, many of these experiments were performed in the presence of serum and serum-component proteins capable of stimulating as well as inhibiting erythropoietic activity (7, 13, 14, 38, 55, 87). Using rigorous serum-free conditions, it has been shown that IGF-1 can substitute for EPO for normal erythroid progenitors and even more effectively for PV erythroid progenitors (13, 55). Furthermore, it has also been observed that anephric patients with no detectable EPO and normal hematocrits have elevated IGF-1 levels (7). Most of the circulating IGF-1 is bound to six known high affinity IGF-1 binding proteins (IGFBP-1 to IGFBP-6). Compared to normal subjects, IGFBP-1 is increased four-fold in patients with PV. IGFBP-1 is also a powerful stimulator of normal and PV erythropoiesis *in vitro* (54, 55). However, while the studies using a single cytokine in a serum-free media improved our knowledge of physiological processes in health and disease, they may also result in misleading conclusions. After all, cells *in vivo* are exposed to multiple cytokines that activate different receptors, some sharing signaling molecules resulting in "receptor cross-talk". This complex interaction of serum factors, receptors, and post-receptor signaling molecules capable of fine control may not be emulated by *in vitro* experiments using cell lines, artificial conditions or non-human tissues.

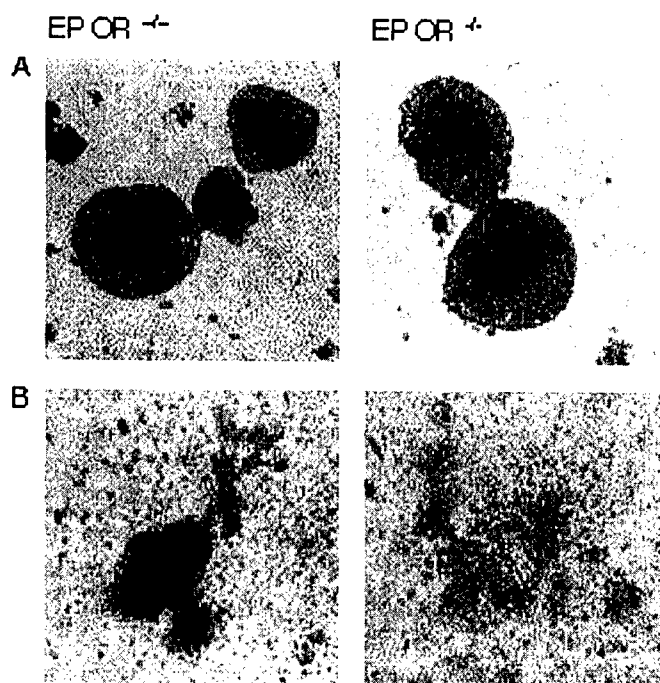


Figure 2. *In vitro* differentiation of *EPOR*^{-/-} and wild-type ES cells in two-step cultures. (A) Embryoid bodies at day 9 of differentiation in α -methylcellulose with serum and SCF. (B) Erythroid colonies derived from disaggregated embryoid bodies. The semisolid media contained serum, SCF and TPO. Both *EPOR*^{-/-} and wild-type colonies expressed endogenous EPO (not shown).

The renin-angiotensin system regulates blood pressure, renal hemodynamics and fluid and electrolyte homeostasis (27). The primary function of angiotensin during development is the modulation of tissue growth and differentiation (28). The growth effects of angiotensin II are dependent on the differentiation state of the cells at the time of exposure to angiotensin II (71). Angiotensin II is a ligand for two distinct receptors, type 1 and type 2. The angiotensin type 1 receptor (AT₁), which is found in liver, lung, adrenal gland, placenta, pituitary gland, aorta, heart, skeletal muscle, lymphocytes, monocytes and platelets, appears to have a major role in the modulation of cell proliferation (95). The therapeutic effects of angiotensin-converting enzyme (ACE) inhibitors and losartan (a specific antagonist of AT₁) on post-transplant erythrocytosis or polycythemia in renal transplant patients indirectly link angiotensin II with the regulation of erythropoiesis (34, 63). We have reported the presence of AT₁ on erythroid progenitors and found that its ligand, angiotensin II, augments EPO stimulation of erythropoiesis (57). The involvement of JAK2 kinase in angiotensin II-

mediated intracellular events suggests that the signal transduction pathways mediated by EPO and angiotensin II may overlap (52).

POLYCYTHEMIAS DUE TO DEFINED MOLECULAR LESIONS

Secondary polycythemia: High affinity hemoglobin mutants, methemoglobinemias, and 2,3 bisphosphoglyceromutase (BPGM) deficiency. Though first defined as a cause of congenital polycythemia, high oxygen affinity hemoglobin mutants are an uncommon cause of congenital secondary polycythemia. More than 50 hemoglobin variants of both α and β globin chains are characterized by an increased oxygen affinity of hemoglobin and lead to autosomal dominant polycythemia (reviewed in Ref. 68). The hemoglobin tetramer oscillates between the R (relaxed; fully oxygenated hemoglobin) and T (tense; fully deoxygenated hemoglobin) states of the quaternary protein conformation requiring the cooperative interaction of globin subunits. Mutations affecting the equilibrium between R and T transition result in a change of oxygen affinity. Many high oxygen affinity mutants have amino acid changes located in the $\alpha 1/\beta 2$ interface of the hemoglobin tetramer. Some mutations interfere with the binding of 2,3-bisphosphoglycerate (2,3-BPG) to hemoglobin; others have an amino acid substitution at the C-terminus of one of the globin subunits, favouring the R state. The functional consequence of increased hemoglobin oxygen affinity is decreased delivery of oxygen into the peripheral tissues and compensatory polycythemia. This assures normal oxygen tissue delivery, thus patients inheriting these mutations are generally asymptomatic. Since many of these mutants are electrophoretically silent, the determination of hemoglobin oxygen dissociation kinetics and P_{50} (partial pressure of O_2 in mm Hg, where Hb is 50% oxygenated) is the best initial screening for suspected congenital secondary polycythemia. If an oxymeter is not available, P_{50} can be mathematically estimated from a venous blood gas measurement (50). Decreased P_{50} indicates either mutant hemoglobin or 2,3 bisphosphoglyceromutase (BPGM) deficiency, a rare cause of increased hemoglobin oxygen affinity (26). Polycythemia due to congenital methemoglobinemia is always associated with easily discernable coexistent cyanosis and as such does not represent a diagnostic dilemma. Methemoglobin is a derivative of hemoglobin in which the ferrous ions (Fe^{2+}) are oxidized to the ferric (Fe^{3+}) state. Oxygen binds reversibly to Fe^{2+} in deoxyhemoglobin but not to methemoglobin. Furthermore, the presence of Fe^{3+} hemes in some subunits of hemoglobin tetramer increases the oxygen affinity of the accompanying Fe^{2+} hemes. A compensatory polycythemia develops ensuring normal oxygen delivery to tissue. There are three types of

hereditary methemoglobinemia: hemoglobin M (Hb M) disease (dominantly inherited due to a mutation of one of the globin genes); NADH-cytochrome b5 reductase deficiency and cytochrome b5 deficiency (reviewed in Ref. 68); the latter two recessively inherited.

Primary familial and congenital polycythemia (PFCP)

PFCP (also called benign erythrocytosis or familial erythrocytosis) is an uncommon cause of polycythemia, but in our experience is a far more prevalent cause of congenital polycythemia than the high oxygen affinity hemoglobin mutants or 2,3-BPG deficiency. Typically inherited as an autosomal dominant disorder, PFCP is characterized by an elevated erythrocyte mass and hemoglobin concentration, hypersensitivity of erythroid progenitors to EPO in serum containing clonogenic cultures, low serum EPO level, normal hemoglobin oxygen dissociation and absence of progression to leukemia (24, 35, 67, 69 and reviewed in Ref. 42). All PFCP-causing mutations described thus far are due to truncation of the cytoplasmic EPOR domain (40, 42, 69). "Serum-free" *in vitro* experiments with myeloid cell lines retrovirally transfected with murine truncated EPOR suggested that the truncated EPOR might, in fact, impede erythropoiesis due to a deletion of docking site initiating a PI-3-kinase anti-apoptotic pathway (18). However, the recent creation of mice bearing normal human *EPOR* and mutant disease-causing human *EPOR* (truncated after the first tyrosine residue of the intracellular domain) suggested that the JAK2/STAT-5 pathway triggers the main antiapoptotic signals mediated by EPOR (21). A polycythemic phenotype, found in both homozygous and heterozygous mice for mutant *EPOR* gene confirmed the principal roles of JAK2/STAT-5 in the positive and SHP1 in the negative regulation of adult erythropoiesis. *In vitro* cultures of erythroid progenitors derived from these animals also proved that the late erythroid progenitor (CFU-E) is the main target for EPO/EPOR regulated signaling.

The effect of a truncated EPOR in the host milieu is not always predictable. When the molecular basis of polycythemia in the index family that led to the original description of the PFCP disease entity (67) was examined, it was initially concluded that *EPOR* was not linked with PFCP since a child in the third generation (who inherited the same *EPOR* haplotype [86] as the affected father, uncle and grandfather) did not have polycythemia. However, this child had the typical *in vitro* erythroid progenitor EPO hypersensitivity seen in PFCP, indicating the presence of unidentified epigenetic factors that masked the full phenotypic expression of the *EPOR* mutation (46).

In some families linkage of PFCP with *EPOR* mutations has been conclusively excluded, further indicating a dominant lesion in an

unidentified gene(s) either at the level of the EPOR signaling pathway or another erythropoiesis regulating pathway (45, 85). To further examine the role of *EPOR* mutations in the pathogenesis of PFCP, we examined 50 unrelated PFCP subjects for *EPOR* and found only five (10%) of the patients had *EPOR* mutations (44). These findings strongly suggest that mutations in *EPOR* are not the major genetic defect in the pathogenesis of PFCP and the gene(s) responsible for the majority of PFCP cases remain to be defined.

POLYCYTHEMIAS WITH INCOMPLETELY DEFINED MOLECULAR LESIONS

Chuvash polycythemia

The only endemic congenital polycythemia is the recessively inherited Chuvash polycythemia (CP) found in the Chuvash people (of Asian ethnic origin) in Central Russia (22, 64, 81, 94). In these individuals, thrombotic and hemorrhagic vascular complications lead to early mortality, usually before the age of 30 years (3). The serum EPO concentrations in the polycythemic patients are significantly higher compared to healthy first-degree family members. The elevated EPO level suggests a secondary polycythemia and therefore one would expect normal responsiveness of erythroid progenitors to EPO. However, the erythrocyte progenitors of persons with Chuvash polycythemia are EPO hypersensitive (3, 4), a feature consistent with a primary polycythemia. Thus, Chuvash polycythemia blurs the distinction between primary and secondary polycythemia. Based on clinical and laboratory data, we hypothesized that CP may be due to dysregulation in the oxygen-sensing pathway, and that HIF-1 α may play a role in the pathogenesis of this disease. Western blot analysis using EBV transformed B-lymphocyte cell lines from CP individuals revealed higher normoxic HIF-1 α protein and mRNA levels in compare with controls. These data indicate that CP is an inherited disorder of hypoxia sensing. However, mutations of *HIF-1 α* , *EPO*, and *EPOR* genes as a cause of CP were ruled out (3, 4) and the involved gene(s) remain to be identified.

Polycythemia vera (PV)

PV is the most common primary polycythemia. As with other myeloproliferative syndromes, PV is caused by an acquired mutation in a single hematopoietic progenitor that leads to an increased erythrocyte mass and variably increased platelets and myeloid cells. While the identity of gene(s) and the nature of the disease causing mutation responsible for PV remains elusive, some cellular biological characteristics of PV have been

established and these should facilitate eventual identification of the PV responsible gene and the elucidation of its molecular pathology (61). Since PV arises from a single hematopoietic progenitor, all or the vast majority of the circulating myeloid cells are clonal (1). The mutation leading to clonal myeloid expansion in PV affects the pluripotent stem cell as a variable proportion of B lymphocytes are a part of the PV clone, however, the majority of the T and NK cells is polyclonal (29). When responses of PV bone marrow or peripheral blood progenitors are tested *in vitro*, PV progenitors form erythroid colonies in the absence of exogenous EPO (10, 65, 98, 99), a phenomenon not observed in normal bone marrow samples (reviewed in Ref. 66). The erythroid colony assay is a useful tool to distinguish PV from secondary polycythemia (66, 69), ET (82) and PFCP (67). Anti-EPO and anti-EPOR neutralizing antibodies completely suppress or abolish the formation of erythroid colonies in normal as well as in PFCP samples, but not in PV samples (25). However, these observations hold true only in the presence of serum. In serum-free culture systems, when compared to normal cells, PV progenitor cells exhibit hypersensitivity to IGF-1 but intriguingly, no differences are observed in responses to EPO (14, 55). In other culture systems, hypersensitivity of PV erythroid progenitors to other cytokines (IL-3, GM-CSF, SCF) was reported, but to a lesser magnitude than to IGF-1 (15, 16). These data suggest that the putative PV defect affects signaling downstream from the cytokine receptors, resulting in increased proliferative responsiveness of PV cells to cytokine stimulation (41). However, we recently observed similar IGF-1 hypersensitivity of native PFCP erythroid progenitors due to truncated EPOR (70) in serum-free but not in serum containing media, suggesting that increased signaling of the IGF-1 signal transduction pathway is unlikely to be the primary PV defect. The serum-free experiments should be interpreted with caution; while they provide valuable insights into the regulation of erythropoiesis, they can not substitute for *in vivo* complex interactions of serum factors with cellular receptors orchestrating fine control of erythropoiesis.

Molecular basis of abnormal cytokine responses of PV progenitor cells

The number of molecules and ligand affinity of EPOR on erythroid progenitors was found to be identical in normal, PV and in progenitors from subjects with PFCP (24). Furthermore, no mutations were found in the coding sequences of *EPOR* of PV subjects (31). Thus abnormalities of the *EPOR* are not directly involved in PV. The putative lesion associated with PV may result in increased tyrosine kinase activity or decreased tyrosine phosphatase activity associated with cytokine receptors (89). Studies of mice deficient in hematopoietic cell phosphatase (SHP-1), a negative regulator of a number of cytokine receptors, suggested that "loss-of-function" mutations of SHP-1 may result in cytokine independent or hypersensitive proliferation

of cells (76). No mutations, however, were found in the coding region of *SHP-1* and the amount of SHP-1 protein was normal in PV granulocytes and highly purified early hematopoietic precursors (2, 6). Other studies have shown that PV erythroid progenitors exhibit up to threefold higher protein tyrosine phosphatase activity compared to normal cells (100). Dysregulation of the expression of a number of other genes, including the thrombopoietin receptor and *Bcl-xL*, has been observed in PV subjects (56, 83). Several other abnormalities in PV include platelet dysfunction (48, 96), differences in oxidative responses of PV neutrophils and monocytes (73), increased platelet derived growth factor mRNA levels in megakaryocytes (36) and increased tyrosine phosphatase in PV progenitors (90, 15), however, to our knowledge none of these abnormalities is specific for PV. All these abnormalities suggest that the PV defect alters a number of cellular functions and is not restricted to cytokine receptor signal transduction only (32).

Two other intriguing possible mechanisms of PV pathophysiology have been recently reported. The *EPOR* gene can produce several mRNA species by alternative splicing (58); one of the resulting peptide products that inhibits EPOR controlled erythropoiesis was recently reported to be absent in some PV patients (11). A second finding of particular interest is persistence of transcription of the *PRV-1* gene, a novel member of the uPAR receptor superfamily (93). The mRNA for this gene was detectable in PV granulocytes but not in normal subjects or in those with secondary polycythemia due to pulmonary disease. Interestingly, the PRV-1 peptide levels were the same in normal and PV granulocytes (93). This gene does not appear to be mutated, and its continuous transcription in the terminally differentiated PV myeloid cells appears to be a secondary phenomenon (Pahl HL, personal communication, November 2000). Nevertheless, if this defect is found to be indeed specific for PV (i.e., not found in PFCP or in congenital high EPO states such as Chuvash polycythemia), this finding could potentially lead to the development of a rapid, specific PV diagnostic test.

In the rare familial cases of PV, the disease is inherited in an autosomal dominant fashion and is typically seen in elderly family members. This suggests that a new mutation is acquired and the disease may result from the loss of heterozygosity. The existence of these families should greatly facilitate a search for PV causing gene mutation(s) (39, 43). While the molecular target of an acquired mutation leading to PV remains to be elucidated, our knowledge of disease specific defects is increasing. The increasing number of recognized instances of familial incidence of PV suggests that in these families the predisposition for PV is inherited as a dominant trait and that PV is acquired as a new mutation that leads to a clonal hematopoiesis. The existence of these families provides a unique opportunity for isolation of the mutations in the gene leading to PV.

CONCLUSION

Molecular defects of genes important for the regulation of oxygen homeostasis are involved in several congenital and acquired disorders. One of the main physiological processes controlled by oxygen availability is the production of red blood cells, erythropoiesis. Knowledge of alterations of this physiological system devoted to sufficient oxygen supply for normal metabolic functions should facilitate our understanding of hypoxia sensing mechanisms.

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Chapter 14

Erythropoietin use and abuse

When physiology and pharmacology collide

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Abstract: The major function of the erythrocyte is to transport oxygen from the lungs to the other tissues, a function ensured by the glycoprotein hormone erythropoietin which couples red cell production to long term tissue oxygen requirements. Tissue hypoxia is the only physiological mechanism for increasing erythropoietin production but there are a variety of mechanisms for its down regulation including hyperoxia, increased catabolism by an expanded erythroid progenitor cell pool, blood hyperviscosity independently of its oxygen content, renal disease and the cytokines produced in inflammatory, infectious and neoplastic disorders. Erythropoietin lack results in severe and often transfusion-dependent anemia but if bone marrow function is otherwise normal, recombinant human erythropoietin therapy can restore the red cell mass and alleviate the transfusion need. However, elevation of the red cell mass by recombinant human erythropoietin is associated with a reduction in plasma volume and in some patients, hypertension is induced. Elevation of the red cell mass is also associated with a reduction in cerebral blood flow. When used to gradually elevate the hematocrit to 36% in anemic patients, recombinant human erythropoietin therapy is usually uneventful. However, when the normal hematocrit level is exceeded, the risk for thrombotic events increases since blood viscosity varies exponentially with the hematocrit. Increasing the hematocrit by autologous blood transfusions can enhance athletic performance in fit individuals and recombinant human erythropoietin administration is an obvious surrogate for autologous blood transfusions. However, paradoxically, its effects are the opposite of those of endurance training, namely a change in red cell mass without an increase in the total blood volume. Thus, the use of recombinant human erythropoietin as a performance-enhancing agent is dangerous, particularly in the less fit athlete, and probably of little benefit in the highly conditioned one. Differences in the carbohydrate content of native and recombinant human erythropoietin are identifiable by isoelectric focusing, providing a direct means for detecting

erythropoietin abuse using urine specimens; a panel of surrogate blood markers of enhanced erythropoiesis such as soluble transferrin receptors, serum erythropoietin, reticulocyte hematocrit and percent macrocytes provide an indirect means for this purpose. Timing of surveillance is, of course, critical due to biological limitations on the physical presence of the hormone. However, education about its dangers may prove to be the most valuable solution to abuse of recombinant human erythropoietin for competitive advantage.

Key words: blood doping, athletics, hemoglobin, hematocrit

INTRODUCTION

At the start of the twentieth century, Landsteiner's discovery of the blood groups made blood transfusion possible. At the end of the twentieth century, the development of recombinant human erythropoietin made it possible to obtain many of the benefits of blood transfusion without transfusing blood. Today, patients with end-stage renal disease no longer require transfusions to alleviate their anemia and bloodless surgery is no longer an oxymoron. Unfortunately, these medical miracles also lent themselves to sinister as well as life-saving practices. Blood transfusion or its surrogate, recombinant erythropoietin are currently being used to enhance athletic performance by improving maximal oxygen transport, a practice that is not only unethical and illegal but also dangerous. In this article the fundamental principles of erythropoietin physiology and pharmacology are reviewed to provide a perspective on the hazards of the non-medical practice of induced erythrocythemia (blood boosting or doping) and the means for detecting it.

ERYTHROPOIETIN PHYSIOLOGY

Normally, the red cell mass is constant in a given individual, but can vary by more than 10% between individuals of the same age and gender. Although apparently static, the red cell mass actually represents a dynamic equilibrium in which red cells lost from the circulation through senescence are replaced on equal basis by newly formed red cells from the bone marrow. The principal function of the red cell is to transport oxygen from the lungs to the other tissues, a function whose importance is underscored by the fact that basal body oxygen consumption is 4 ml/kg/min. while total body oxygen stores are only 20 ml/kg. Red cell production is regulated by the glycoprotein hormone erythropoietin. Like the red cell mass, under normal circumstances the plasma erythropoietin level is also constant in a given individual, although the level can vary more than 2 fold amongst

different individuals without relationship to age, gender or even the red cell mass (84). The principal function of erythropoietin is to couple red cell production to long term tissue oxygen requirements.

Erythropoietin, which is one of the few hematopoietic growth factors that acts like a hormone, is uniquely suited to its function. Produced constitutively primarily in the kidneys in adults and to only a small extent in the liver, erythropoietin acts on its target cells in the bone marrow as both a mitogen and a survival factor. It triggers early erythroid progenitor cells that are largely dormant into cell cycle (87) and maintains the viability of late erythroid progenitor cells, which are largely cycling, while they differentiate (43). Like most hematopoietic growth factors, this latter behavior of erythropoietin is permissive since immortalized red cell progenitors do not require erythropoietin for terminal differentiation (79). The mitogenic effect of erythropoietin is, however, both instructive and permissive. Erythropoietin acts as both a competence factor in cell cycle initiation and a progression factor for entry into S phase (85). The different effects of erythropoietin require different concentrations of the hormone. Thus, for dormant early erythroid progenitor cells that express few erythropoietin receptors, a high concentration of erythropoietin is required; for actively cycling late erythroid progenitor cells that express many erythropoietin receptors, only a small amount of the hormone is required (35,70). This differential requirement is reflected in the relatively low and constant plasma erythropoietin level characteristic of its constitutive production and the marked but usually transient increase in plasma erythropoietin associated with tissue hypoxia (1).

Erythropoietin Production

Erythropoietin production is regulated at the level of its gene (33). Hypoxia is the principal environmental stimulus for upregulating erythropoietin production. In the interstitial fibroblasts of the kidneys where erythropoietin is synthesized, production is constitutive; it is also maximal and an increase in erythropoietin production is achieved by the recruitment of additional renal interstitial cells to produce the hormone (45). In the liver, hepatocyte erythropoietin production is usually submaximal and the hypoxic stimulus required for its upregulation is much greater than in the kidneys (44). An additional mechanism for increasing erythropoietin production appears to involve intracellular iron stores since under conditions of iron deficiency, erythropoietin production is upregulated (41), but the consequences of this mechanism must be minimal given that an adequate iron supply is essential for erythroid progenitor cells to respond to the hormone. As a reciprocal effect, erythropoietin upregulates transferrin receptor production by stabilizing its mRNA (95).

In contrast to the limited mechanisms for upregulating erythropoietin production, there are multiple mechanisms for down regulating it. The first involves increased tissue oxygenation. Thus, an absolute increase in tissue oxygenation such as provided by oxygen administration or blood transfusion will suppress erythropoietin production at the level of gene transcription even with severe anemia (93). As a corollary, a decrease in tissue oxygen needs such as in hypothyroidism or panhypopituitarism will also result in a reduction in erythropoietin production. Erythrocytosis occurring as compensation for tissue hypoxia also suppresses erythropoietin production if the hypoxic stimulus is not severe (56). This is in part, the basis for the usually normal plasma erythropoietin level in patients with cyanotic congenital heart disease (36). It is also in part, the basis for the very low plasma erythropoietin level characteristic of polycythemia vera (42).

An increase in blood viscosity is a second mechanism for decreasing erythropoietin production (80). Although the biologic basis for this is undefined, it appears to be independent of tissue hypoxia (40). This mechanism may also account in part for the normalization of plasma erythropoietin in patients with cyanotic congenital heart disease as well as in patients with an increase in blood viscosity due to hypergammaglobulinemia. In the latter group of patients, the resulting anemia also serves to reduce blood viscosity. The marked reduction in plasma erythropoietin in uncontrolled polycythemia vera also reflects in part, the increase in blood viscosity that occurs when this disorder is uncontrolled.

A third mechanism for regulating the plasma erythropoietin level is increased catabolism. The major mechanisms for erythropoietin catabolism remain to be defined as only 5-10% of the hormone is lost intact in the urine (24) but some of the hormone is catabolized within its target cells (73) and with an increase in the number of erythroid progenitor cells, the rate of catabolism increases. This was seen most clearly in the shortening of the plasma clearance of the erythropoietin after repeated injections (54) and in the reduction in plasma erythropoietin that occurred in iron-deficient patients given iron before there was any increase in the hematocrit level (54). Increased catabolism undoubtedly also explains some of the reduction in plasma erythropoietin in patients with polycythemia vera and secondary forms of erythrocytosis. As a corollary, the very high plasma erythropoietin levels seen in patients with aplastic anemia, red cell aplasia or myelodysplasia probably reflect not only tissue hypoxia but impaired hormone catabolism due to a contracted or dysfunctional erythroid progenitor cell pool.

A fourth mechanism for down regulating erythropoietin production involves cytokines such as IL-1, TNF α and the interferons. These agents suppress erythropoietin production as well as suppressing the ability of erythroid progenitor cells to respond to the hormone (26,38,55). Since production of these cytokines is a feature of inflammation, infection and

cancer, it is not surprising that anemia commonly develops in these situations.

Direct tissue damage is a fifth mechanism for reducing erythropoietin production. This finds its most frequent expression in parenchymal renal disease but can occur secondary to drug or toxin exposure. In chronic renal failure states, the expected inverse relationship between the plasma erythropoietin level and the hematocrit is lost (31), as is the ability to sustain an increase in erythropoietin production due to an hypoxic stimulus (15). This is most likely due to the insensitivity of hepatic cells to hypoxia.

It is worth noting that androgens have little to do with erythropoietin production. Thus, men and women have different red cell masses but the same range of plasma erythropoietin levels (84). Castration leads to a decline in hemoglobin without a change in the plasma erythropoietin level (94). In this regard, the degree of anemia required to significantly upregulate erythropoietin production when renal function is normal is substantial. Thus, until the hematocrit falls below 10.5 gm%, the plasma erythropoietin level does not increase outside the normal range (84).

Given the significance of the consequences of inappropriate elevation of the red cell mass, hyperviscosity, circulatory overload and thrombosis, it is not surprising that erythropoietin production is tightly regulated. As corollary, its clinical use needs to be equally judicious. This is not merely because an inappropriate increase in the red cell mass can be deleterious but also because of the mechanisms by which erythropoietin elevates the red cell mass. The hormone does not merely increase the number of circulating red cells; it also reduces the plasma volume. In deed, in any situation in which there is erythropoietin excess, endogenous or exogenous, as the red cell mass increases, the plasma volume decreases. This phenomenon, while unexplained, is encountered in patients with cyanotic congenital heart disease (36), chronic mountain sickness (68), chronic carbon monoxide intoxication (81), androgen use (5) and with recombinant human erythropoietin administration (47). It is also observed with blood transfusion (71), suggesting that it is the change in red cell mass that is essential not the mechanism for the change. Unfortunately, little attention has been devoted to this important physiologic response which has significant pathophysiologic implications since at its most extreme, the normal relationship between the plasma volume and the red cell mass is reversed.

Plasma Erythropoietin

The availability of the recombinant erythropoietin led to the development of a sensitive and specific immunoassay for the hormone in biologic fluids, thereby permitting for the first time an accurate means for studying its physiology in vivo (19). The immunoassay for erythropoietin has proved

useful for the following reasons (84): 1) there is only one form of circulating erythropoietin; 2) the hormone is biochemically unique; 3) immunoreactive circulating erythropoietin and biologically-active circulating erythropoietin are equivalent; 4) in the absence of hypoxia or disease, plasma erythropoietin is constant in a given individual; 5) the half-life of plasma erythropoietin is long and independent of its plasma concentration; 6) erythropoietin production is regulated at the level of its gene; 7) tissue hypoxia is the only physiologic stimulus for erythropoietin production; 8) there are no preformed stores of the hormone and 9) there is an inverse correlation between the plasma erythropoietin level and the hemoglobin or hematocrit.

Since erythropoietin behaves like a hormone and tissue hypoxia is the stimulus for its production, assay of plasma erythropoietin should provide a means of determining if synthesis of the hormone is appropriate in an anemic individual. This, in deed, proved to be the case. In addition to intrinsic renal disease, a number of other conditions such as infection, inflammation and cancer were identified in which there was an inappropriately low plasma erythropoietin level for the degree of anemia (83) and as such, potential situations in which recombinant erythropoietin could be used to alleviate symptomatic or transfusion-dependent anemia.

RECOMBINATION HUMAN ERYTHROPOIETIN THERAPY

Since the kidneys are the major site of erythropoietin production, proof of principle was first sought in anemic patients with end-stage chronic renal disease. These patients proved to be uniformly responsive to treatment with recombinant human erythropoietin with alleviation of transfusion-dependence and correction of anemia in 97% (25). Recombinant erythropoietin proved to be remarkably safe if used judiciously. Hypertension was the major toxicity encountered with either exacerbation of preexisting hypertension or induction of de novo hypertension in approximately 40% of patients receiving it. Notably, the increase in blood pressure usually occurred in the first 8 weeks of therapy in hematocrit (67). Since administration of large quantities of recombinant erythropoietin did not cause hypertension in normal volunteers, it is unlikely that hypertension was a direct effect of erythropoietin (13). Furthermore, in patients without renal disease, hypertension as a consequence of recombinant erythropoietin therapy is uncommon if inappropriate elevation of the hematocrit is avoided. Patients with end-stage kidney disease appear to represent a unique population since they also respond to blood transfusion by increasing their blood pressure (59). Although the exact mechanism for erythropoietin-induced hypertension is still undefined, several possibilities exist. First, for

unknown reasons exposure to increased quantities erythropoietin, either endogenously or exogenously, is associated with a diminution of the plasma volume. Second, and possibly the basis for the fall in plasma volume, is erythropoietin-induced vasoconstriction, the mechanism for which is not yet resolved since it does not appear to be due to changes in the red cell mass but may involve resistance to NO and changes in cytosolic calcium in smooth muscle cells (91).

Patients with end-stage renal disease, of course, constitute a unique group because of the high incidence of preexisting vascular disease, hypertension and fluid balance problems. They also constitute a unique group with respect to erythropoietin therapy since their bone marrow function is usually normal except for the effects of erythropoietin deficiency and their often precarious iron balance. It is of interest, therefore, that subsequent therapeutic trials of recombinant human erythropoietin for correction of anemia associated with other diseases were based on the dosing, route and timing of recombinant erythropoietin administration in end-stage renal disease patients. Although the situation is now different, initially recombinant human erythropoietin was given intravenously three times weekly, a route and schedule of administration that coincided with the dialysis schedule and drug delivery approach used for the renal failure patients. Today, recombinant human erythropoietin is administered to anemic patients without renal disease subcutaneously once a week at a standard dose of 40,000 units. In patients with renal disease, a similar approach is now used but with smaller doses to avoid exacerbating hypertension or triggering it.

Erythropoietin administered subcutaneously has a more favorable pharmacokinetic profile than when administered intravenously (50). Intravenously administered erythropoietin has a complex plasma clearance that is best explained by a two-compartment model with an initial distribution phase followed by a slower elimination phase (86). In humans, the half disappearance time for the hormone is 8 ± 2 hours (54). By contrast, after subcutaneous administration of the hormone, entry of the hormone into the circulation is slow but in contrast to intravenous administration, its plasma residence is sustained with a half life of 48 hours, resulting in greater bioavailability. In deed, in patients with end stage renal, erythropoietin administered subcutaneously is a third more potent than the equivalent quantity of hormone administered intravenously (39).

In contrast to anemic patients with end-stage renal disease, patients with anemia due to other causes but with normal renal function respond to the hormone only 60% of the time and for unknown reasons, doses of erythropoietin greater than 40,000 units given once weekly or in divided doses are often not more effective (18). Iron therapy is not required in iron-replete patients, defined as having a serum ferritin of greater than 100 ng/ml if renal function is normal or 200 ng/ml if renal function is impaired (66). A plasma erythropoietin level greater than 500 U/ml in an anemic patient also

suggests an unresponsive bone marrow that is not likely to respond to exogenous erythropoietin (37). Failure to achieve at least a 0.5 gm % increment in hemoglobin after 2 weeks of therapy is another indication of an unresponsive bone marrow (49).

Quality of life studies have firmly established that alleviation of anemia by recombinant erythropoietin is associated with a reduction in fatigue and an improvement in exercise tolerance. This is as true in anemic cancer patients (18) as it is in patients with end-stage renal disease (53). However, in the former population, the benefits seem to be most marked in women (18). This may be a reflection of the reluctance to increase the hemoglobin level greater than 12 gm%, a level at which all men will be still be anemic.

INDUCED ERYTHROCYTHEMIA: AUTOLOGOUS BLOOD TRANSFUSION

The ability of recombinant human erythropoietin to alleviate anemia did not go unnoticed by those concerned with increasing tissue oxygen transport for the purpose of improving athletic performance. The principle that increasing the red cell mass improved exercise tolerance was established 54 years ago (61) and subsequently confirmed in a number of well-controlled studies that emphasized the positive effect of induced erythrocythemia on oxygen transport and maximal aerobic capacity. First, it was demonstrated that there was a close relationship between maximal oxygen uptake (Vo_2 max) and the hemoglobin level within the physiologic range of hemoglobin concentrations such that a reduction in red cell mass without induction of anemia resulted in a reduction in Vo_2 max while an increase in red cell mass within the range of normal as well as above it was associated with an immediate increase in Vo_2 max and work endurance (11,14,22). Thus, a normal red cell mass, regardless of whether it was at the high end of normal or the lower end, was not optimal in terms of maximal oxygen uptake. Of particular interest was the observation that even after a return to a normal hemoglobin level, elevations of the Vo_2 max persisted, suggesting an undefined conditioning benefit (11). The basis for the initial blood transfusion-associated increase in Vo_2 max was, however, clearly demonstrated to be due to an increase in blood arterial oxygen content since changes in cardiac output, minute ventilation and venous blood oxygen content were minimal (22,90). Importantly the effectiveness of induced erythrocythemia in increasing the Vo_2 max was directly related to the fitness of the individual. Unfit individuals or very fit individuals benefited less than those of moderate fitness as defined by initial aerobic power between 50-65 ml/kg/min (72).

Second, the benefits of induced erythrocythemia on blood oxygen content were as evident on the playing field as in the physiology laboratory. Thus, the reinfusion of 400 ml of red cells in highly trained runners improved their performance time in a 10 kilometer race by approximately one minute and this improvement in performance was sustained for 13 days (9). There was no evidence that the observed effects were caused by a change in heart rate, shift in hemoglobin P_{50} or 2,3-DPG and the apparent final hematocrit following the reinfusion of blood was well within the normal range (9). Similar observations have been made in cross-country skiers, including the sustained conditioning effect (4).

Recombinant Human Erythropoietin as a Surrogate for Blood Doping

As mentioned above, the great benefit of recombinant erythropoietin is that it can provide many of the benefits of blood transfusion without transfusing blood. The success of recombinant erythropoietin in the clinical arena was not lost on those participating in the athletic area. Induced erythrocythemia (or blood boosting or doping) through the transfusion of stored autologous blood is a cumbersome procedure, the effect of which could be much more easily achieved through a series of injections of recombinant human erythropoietin. Once again proof of principle was provided by controlled experiments in which intermittent low dose recombinant erythropoietin therapy (20-40 U/kg T.I.W) increased the red cell mass and VO_2 max in a manner equivalent to transfusion-induced erythrocythemia (2). However, in contrast to the latter, recombinant erythropoietin-induced erythrocythemia was accompanied by an increase in systolic blood pressure with exercise although diastolic blood pressure did not increase significantly (2).

The Pathophysiology of Induced Erythrocythemia

There are no controlled studies of the impact of administration of recombinant erythropoietin on performance in specific types of endurance events but there are many anecdotal reports of wide spread use of the recombinant hormone in the international sports community including rumors of fatal consequences as a result (20). That recombinant erythropoietin was so rapidly embraced says much not only about the assumed effectiveness and inconvenience of blood doping but also about the lack of understanding of erythropoietin physiology and the adaptive physiology of endurance conditioning.

Well-conditioned athletes differ from their unconditioned counterparts with respect to the size of their blood volume, their cardiac stroke volume

and their heart rate during exercise. Conditioning leads to some (75) or no increase in red cell mass (34) but a significant increase in plasma volume (34,60,75). Total blood volume is also increased. This results in a decrease in peripheral vascular resistance and at any given hematocrit level cardiac output and systemic oxygen transport are increased when the total blood volume is expanded (58). Since an increase in blood volume is a more efficient means of increasing cardiac output than increasing the heart rate, cardiomegaly and bradycardia are the consequences of effective conditioning (23,32). The increase in plasma volume is due to an increase in albumin synthesis and not only is the intravascular volume increased, but extravascular volume is as well (65).

In contrast to the effects of optimal athletic conditioning, physiologic compensation for tissue hypoxia, as indicated above, is associated with an increase in the red cell mass and a reduction in the plasma volume. This type of response also occurs following blood transfusion (71). However, this has never been appropriately emphasized in the literature on induced erythrocythemia because independent measurements of the red cell mass and plasma volume are not usually obtained. Rather single measures of either the plasma volume or red cell volume have been made with extrapolation of the other using the hematocrit. This approach is simply erroneous since there is no fixed relationship between the venous (or arterial) hematocrit and the actual red cell mass: plasma ratio of the blood as a whole (88). Furthermore, the red cell mass and plasma volume can vary independently. Finally, it also needs to be remembered that the hematocrit within various organs not only differs but also is generally lower than that of the peripheral venous hematocrit. For example, the hematocrit in resting skeletal muscle is 20% while that of the spleen is 80% (30). Thus, conclusions based on in vitro measurements of whole blood viscosity and observed or indirectly calculated hemoglobin or hematocrit levels may have no relevance to the actual situation in vivo (14). This appears to be particularly valid with respect to the concept of an optimal hematocrit about which it has been stated "that the optimal packed cell volume for oxygen transport is the highest that can be obtained" (17). When in fact the data upon which this conclusion was based were neither derived from an absolute determination of the total body hematocrit nor extended to hematocrit values significantly outside the range of normal. The more accurate conclusion is that for any given total blood volume, peak systemic oxygen transport actually occurs within the normal hematocrit range (58).

Given these considerations, it is clear that an artificial increase in the red cell mass either by transfusion or by administration of recombinant human erythropoietin is usually associated with a decrease in the plasma volume and no change in total blood volume. This represents an unphysiologic situation particularly in the setting of increased physical activity with the attendant need for increased heat transfer and obligate fluid loss. Not

surprisingly experimental subjects receiving recombinant human erythropoietin had a significant increase in blood pressure with strenuous exercise (2). Thus, induced erythrocythemia by any technique is potentially dangerous but given the less predictable effect of recombinant erythropoietin on the red cell mass and plasma volume as compared with autologous blood transfusion where the actual added volume is known, the use of recombinant erythropoietin is more hazardous.

Detection of Induced Erythrocythemia: The Use of Markers of Erythropoiesis

Induced erythrocythemia when employed to improve athletic performance is both unethical and dangerous and has been banned by both national and international sports authorities. Effective enforcement of such a ban however requires the unequivocal identification of offenders; a substantially difficult task given the physiology of induced erythrocythemia. The ergonomic benefits of blood doping persist for at least two weeks after autologous transfusion while the limited biochemical effects of the procedure, elevations of serum iron and bilirubin were transient (4). Serum erythropoietin was depressed but not outside the range of normal, necessitating a baseline sample to identify the reduction. Furthermore, training alone by increasing the plasma volume, reducing blood viscosity and increasing the cardiac output will also result in a reduction in plasma erythropoietin. Since there is no direct correlation between the hematocrit or hemoglobin and the red cell mass or even a need to elevate the red cell mass outside the range of normal to achieve an ergonomic benefit (11,14,22,90), serial hematocrits would be required to detect any change. The greatest deterrent to blood doping therefore, lies not in testing procedures but rather in the ease with which recombinant erythropoietin can accomplish the same effect.

A number of different approaches have been employed to detect the illicit use of recombinant human erythropoietin. Pharmacologic acceleration of erythropoiesis is accompanied by distinct quantitative and qualitative changes in erythrocytes and serum proteins. Administration of pharmacologic doses of recombinant erythropoietin results in increases in serum erythropoietin, reticulocyte number, hypochromic erythrocytes, reticulocytes, red cell MCV, hypochromic reticulocytes and soluble transferrin receptors, and decreases in reticulocyte hemoglobin, serum ferritin, and transferrin saturation (3;8,10,12,27). It is important to emphasize that the changes reticulocyte or erythrocyte hemoglobin content as well as serum ferritin are dependent on the magnitude of the stimulus (dose of erythropoietin) and body iron stores. Thus, the administration of intravenous iron or adequate body iron stores can blunt the expected response (7,8,51). Furthermore, the timing of observations is critical since once the erythropoietic stimulus was removed, many of the various changes

dissipated from 10-14 days (28,29,51,89). However, post erythropoietin therapy, certain changes characteristic of exposure to the hormone were identified. These included a persistent elevation of the hematocrit and a reduction below normal of the reticulocyte hematocrit and the serum erythropoietin level (62).

Interestingly, recombinant erythropoietin therapy but not exercise alone was also associated with an increase in urine fibrin degradation products (7,82), possibly secondary to an increase in circulating t-PA activity (62). The utility of this with respect to the detection of illicit erythropoietin use is still undefined.

Based on the observed behavior of various markers of induced erythropoiesis during recombinant erythropoietin administration, several panels of these markers have been identified that are indicative of exposure to the hormone (51). A combination of serum erythropoietin, reticulocyte hematocrit, soluble transferrin receptor, hematocrit and percent macrocytes identified current erythropoietin users with an accuracy of 94-100% with only one false positive result (51). A test panel including hematocrit, reticulocyte hematocrit and serum erythropoietin identified erythropoietin exposure in 67-72% users up to 21 days after the last exposure without any false positives but with a progressive loss of sensitivity. A useful feature of multiple marker panel was the inability of a isolated marker to cause a false positive result (51).

This is not a trivial consideration. For example the serum ferritin can vary with the training load (52) and altitude training alone can temporarily reduce serum ferritin while increasing the reticulocyte count (64). Furthermore, although serum erythropoietin levels may or may not change after an endurance event, urine erythropoietin increased (63,77). Intravascular hemolysis, gastrointestinal and urologic hemorrhages are other well-documented effects of strenuous and prolonged exercise (78) and can lead to macrocytosis and iron deficiency (16). Finally two sports authorities, the International Cycling Union (UCI) and the International Ski Federation (FIS), in stipulating an upper limit for the hematocrit (50%, UCI) or hemoglobin (18.5 gm %, FIS) (13), ignored the many dangers of relying on a single test. First, the hematocrit level is subject to change with posture, strenuous exercise, altitude and fluid ingestion (74). Second, the hematocrit does not correlate with the red cell mass. Third, an elevated hematocrit could represent an innocent physiologic variation (76,92) or genetic mutation (48). For example, the hematocrit will vary with place of residence (92) and one Olympic cross-country skiing champion had erythrocytosis secondary to an erythropoietin receptor mutation (48). Finally, the stipulated hematocrit and hemoglobin cut off levels are higher than those that need to be achieved for improved endurance by induced erythrocythemia using any technique (14).

Analysis of Urine Erythropoietin

The use of indirect markers of erythropoiesis relies on blood sampling, timed surveillance, baseline measurements and population-specific controls (6). Furthermore, the influence of iron status and alternative routes of erythropoietin administration on the sensitivity of this approach has also not been established. A more promising approach involves analysis of the electrophoretic behavior of urine erythropoietin. Erythropoietin is a well-conserved protein on an evolutionary basis as befits its vital role in the body's economy. Although there are silent polymorphisms (57), the native and recombinant polypeptides are immunologically indistinguishable (19). However, erythropoietin is heavily glycosylated and the type and extent of glycosylation differs between renal erythropoietin and the commercially available recombinant protein produced in CHO cells (69). The most significant difference involves the extent of sialation. Native erythropoietin and particularly the form excreted in the urine are more heavily sialated than the recombinant protein. As a consequence, the two types of erythropoietin migrate differently when subjected to isoelectric focusing (96). By combining isoelectric focusing with immunoblotting, it has been possible to distinguish recombinant from native erythropoietin (46). Although total urine erythropoietin excretion as measured by immunoassay was not different than normal 4 days after subcutaneous administration of 14,000 U of the hormone (89), what is currently unknown is the duration of excretion of exogenous erythropoietin as detected by isoelectric focusing or the sensitivity of this approach. Importantly, while urine erythropoietin excretion is increased post exercise (63,77), analysis by protein charge avoids false positive results.

Recently, analysis of changes in markers of erythropoiesis was combined with analysis of urine erythropoietin species to improve the specificity and sensitivity of detection of erythropoietin abuse (21). While this is an appropriate approach, more data are needed with respect to confounding variables such as iron deficiency, iron supplementation and routes and timing of erythropoietin administration. This approach also poses a substantial logistical burden, particularly if the responsibility for testing is not decentralized.

SUMMARY

Induced erythrocythemia clearly improves athletic performance but its value for the most fit athlete has not been established. However, the adverse consequences of this practice are unequivocal since the physiology of erythropoietin-induced erythrocytosis is the opposite of exercise-induced

conditioning. The latest strategies to detect erythropoietin abuse have yielded promising results. Even so, testing will probably be insufficient to solve the problem of induced erythrocythemia. The most promising approach is one that enlists the athletes themselves in a proactive partnership with their governing bodies in a program offering education as well as testing. Such a program has been instituted in Italy (13).

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Chapter 15

Mountaineering in thin air

Patterns of death and of weather at high altitude

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Abstract: An 8000-m peak bring challenges of extremes of hypoxia and weather as well as the normal hazards of climbing itself. These challenges have taken a severe toll: 604 mountaineers have died on those great peaks since 1950. Little is known about whether mountain height, use of supplemental oxygen, or team size might influence rates of death or of success. However, such information may provide insights not only to our understanding of the limits of human performance, but also to mountaineers in making decisions on these peaks. We present several examples from a research program that is attempting to analyze factors that potentially influence success or death rates on the 8K peaks. (1) Apparent risk of death in the notorious Khumbu Icefall on Mt. Everest has declined dramatically in recent years. This decline could reflect improved route finding and technique, but might also reflect climate warming, which has caused the Khumbu glacier to shrink and slow in recent decades. (2) Risk of death during descent from an 8000-m peak increases with the height of the peak. (3) Risk of death during descent from the summit of Everest or of K2 is elevated for climbers not using supplemental oxygen. (4) We outline some new studies that are exploring how convective heat loss, which influences wind chill, changes with altitude as well as the incidence of storms: both factors will impact the probability success and death of Himalayan mountaineers.

Key words: Everest, K2, supplemental oxygen, wind chill, convection

INTRODUCTION

Each year thousands of mountaineers venture to the 8000-m peaks of the Himalaya in pursuit of adventure. Each year, some reach a summit; and each

year, a few die. Indeed, a mountaineer's odds of success and of death are demonstrably worse than on lesser peaks (8).

What factors influence the probability of success – and that of death – on the 8K peaks? Remarkably little is known about this issue, despite the extraordinary attention that Himalayan mountaineering inevitably attracts. In fact, prior compilations of quantitative patterns of success and death on the 8K peaks (or any peaks for that matter) are scant. Pollard and Clark (17) were the first to test a specific hypothesis (the probability of death from medical causes would increase with altitude). Recently, we tested the hypothesis that death rate during descent from the summit of Everest or K2 is reduced for climbers using supplemental oxygen than for those using ambient air (4,7). These analyses show that quantitative patterns can be detected from mountaineering data (4)

Here we describe several examples of our research program, which is attempting to analyze factors that might influence the probabilities of success and of death on the 8K peaks. For example, we are exploring whether behavioral choices that a mountaineer makes (e.g., to use supplemental oxygen, to climb alpine style, to climb in winter) influence success and death rates. Similarly we attempt to analyze certain environmental factors (summit height, route steepness, weather) that might also influence those rates. However, we are not currently examining the possible impact of physiological or genetical differences among climbers (11,14).

The general approach

Analyses of Himalayan mountaineering are inherently based on historical data. Experimental approaches, in which one experimentally manipulates one or more variables of interest, are simply not an option (8). Instead, we use a hypothesis-based approach that is rooted in deductive logic (27). Thus we start from an established base of physiological information and derive specific, physiologically plausible predictions. For example, knowing that supplemental oxygen enhances physiological performance at altitude (15), we predicted that death rate during descent from the summit of a high peak is reduced for climbers that use supplemental oxygen (7). We then compile relevant historical data and conduct statistical analyses.

Significant statistical support -- even for an a priori hypothesis -- does not, of course, necessarily imply cause and effect (27): confounding factors can easily distort analyses. This is, of course, a classical problem in epidemiology (24). Accordingly, we attempt to evaluate the robustness of any observed support by considering alternative factors that might confound observed statistical associations. However, as is described below, such evaluations are unfortunately not always feasible or at least easy. Therefore,

we try to be candid as to known uncertainties and alternatives. Readers can then evaluate for themselves the plausibility of cause and effect.

Are statistical approaches useful?

Before reviewing specific analyses, we want to address a fundamental question, namely, do statistics even have place in mountaineering? Some people may feel that statistical analyses are an inappropriate academic intrusion into what should be a wilderness experience. Others may feel that any conclusions drawn from such an analysis are inherently suspect, given that all such analyses are historical and thus non-experimental. We recognize these concerns, but we feel that patterns derived from statistical analyses are still informative, though not definitive.

To illustrate the utility of a quantitative approach, we briefly describe two examples from mountaineering on Mt. Everest. One shows that a statistical approach can sometimes contradict conventional wisdom that is popular but nonetheless patently false. A second presents an example of a generalization that might once have been correct during the formative years of climbing on Everest, but that is no longer correct.

A widespread assertion, seen commonly both in newspapers and even in JAMA (5), is that one in five (or even one in four!) climbers die on Mt. Everest. In fact, the death rate for mountaineers (exclusive of porters, Sherpas, and commercial guides) is actually about one in 48 (R. Salisbury and E. Hawley, personal communication), an order of magnitude lower! In this example a quantitative approach corrects an obvious error that might well have caused considerable anxiety to family and friends of mountaineers. [How could such an obvious error have started? Most likely, the one in five "death rate" probably derives (20) from computing the ratio of the number of climbers who have died *anywhere* on Everest relative to the *number of climbers who have summited*. This ratio may be of interest, but it is certainly not a death rate, which is number of climbers who have died divided by *the number who were at risk on the mountain*.]

A second example concerns the infamous Icefall of the Khumbu Glacier. The Icefall has long been regarded as the most terrifying and dangerous part of the route: even a key history of Everest stated that more deaths have occurred in the Icefall than elsewhere on the mountain (26).

Is the Icefall really the most deadly part of the normal route on Everest? In the early decades, most deaths were indeed in the Icefall; but only one death has occurred in the Icefall since 1987 (R. Huey, A. Salkeld, J. Edwards, E. Hawley, and R. Salisbury, in preparation)! Because *many* people now pass through the Icefall each year, the current death rate – even on a per climber basis – must be near zero. In contrast, most of the recent deaths have occurred on the SE Ridge, even though traffic on that ridge is

miniscule relative to that through the Icefall. Thus, although the Icefall is still undoubtedly dangerous, it is far from the deadliest section on the mountain.

In this example, a quantitative analysis shows that a widespread view, which might once have been valid, is no longer so. Knowing that the SE Ridge, not the Icefall, is the deadliest section should be very relevant to mountaineers. In effect, the Icefall's reputation (26) is now a red herring, and so may put lives at risk.

Why has risk dropped in the Icefall? We can offer two possibilities. First, better route finding, equipment, and technique almost certainly play a role. Indeed, the Icefall route is now maintained by "Icefall Doctors," who charge climbers for access (9)! Second, climate warming may be indirectly responsible. Himalayan temperatures has been warming for several decades (Figure 1), and the Khumbu glacier in particular has been shrinking and slowing (13,19). Perhaps the slowing of the glacier stabilizes the route and reduced risk of death from serac fall or of glacial avalanches.

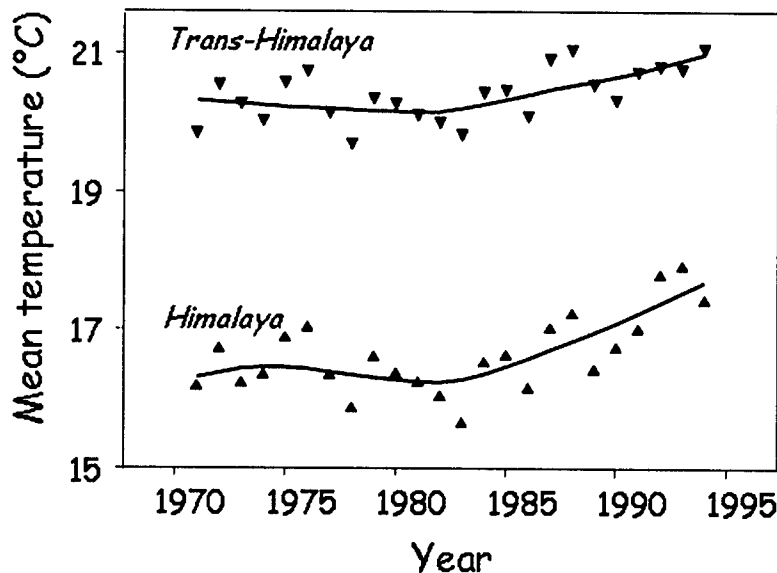


Figure 1. Climate warming in two mountain regions of Nepal. Data courtesy of A. B. Shrestha (21)

This analysis shows that historical statistical analyses can detect pattern. However, it also highlights a basic limitation, namely, the difficulty of evaluating competing processes that potentially underlie that pattern (27). Of course, to a mountaineer attempting Everest, pattern counts: process may be academic.

Mountain height and death rate

Mountaineers are preferentially attracted to the highest summits of the Himalaya, especially to Everest (6). However, because the severity of hypoxic stress (15,28) and of storms (16) increases with the altitude of those summits, risk of death might increase with summit height. Overall risk of death is known so far for only a few 8K peaks (8), but death rates during descent from the summit are now known for all of 14 of the 8K peaks. Consequently, we can test the physiologically based prediction that death rates during descent from the summit increase with the height of a peak.

We analyzed data for all mountaineers who reached the summit of an 8,000-m peak through 2000. On the two highest peaks (Everest and K2), mountaineers commonly use supplemental oxygen (7), which reduces hypoxic stress (15), effectively lowers the “physiological” height of a peak (10) and which is associated with reduced death rates (7). To help standardize comparisons, we therefore excluded data on climbers on these two peaks who used supplemental oxygen. Some climbers who used supplemental oxygen on the other peaks will be included, but hopefully they should be relatively few. We then regressed descent death rate (angular transformed) on altitude (one-tailed test).

Through 2000, 3803 ascents were made on the 8,000-m peaks (range 98 to 1211 ascents per peak, *excluding* O₂-ascents on Everest and K2). Death rate during these descents averaged 3.8% (~ 1 in 26) and ranged from 0.4% on Cho Oyu to 17.3% on K2 (Figure 2). Death rate increased significantly with altitude (Figure 2, $P = 0.009$, $R^2 = 0.39$), even though the maximum difference in altitudes is ~ 800m (Gasherbrum II vs. Everest).

We checked several potentially confounding factors that might cause a spurious correlation between summit altitude and death rate. Reassuringly, altitude remains significant even if data for climbers using supplemental oxygen on Everest and K2 are included ($P = 0.044$) or even if nine K2 climbers that were killed in fierce storms in 1986 and 1995 are excluded ($P = 0.018$). [Thus, the pattern is not an artifact of two severe storms that trapped summit climbers on K2.] Also, descent death rate might increase with summit altitude not because of altitude per se, but perhaps high peaks are farther from base camp, thus prolonging a descending climbers exposure. However, elevational difference between base camp and summit was not significant ($P = 0.18$).

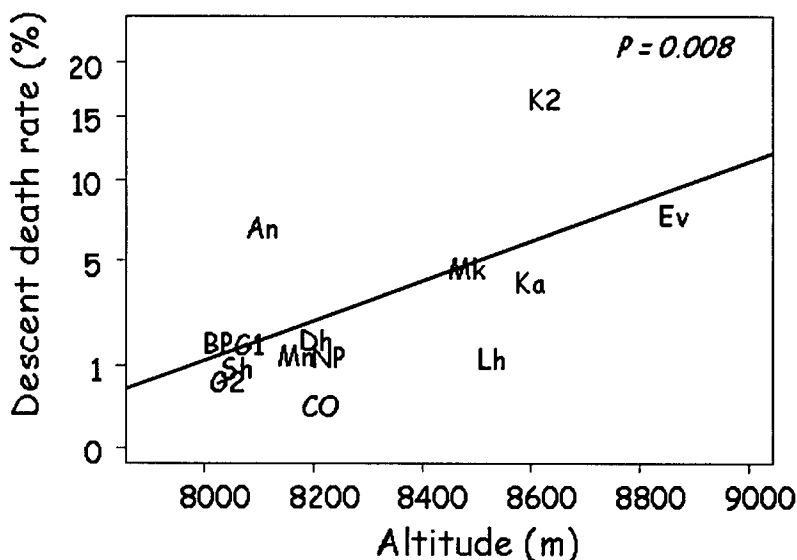


Figure 2. Death rates during descent (through 2000) in relation to the height of each 8000m peaks (symbols are abbreviations for peak names)

Although we can exclude some potentially confounding factors, we can't exclude some factors (e.g., steepness, rock quality, avalanche risk, climber skill and behavior) that might co-vary with altitude: consequently, additional studies will be required to elucidate whether altitude per se is actually the dominant causal factor. Unfortunately, the data necessary to evaluate such alternative factors will be difficult to compile.

Altitude can't be the only factor influencing descent death rates. Annapurna is relatively small but nonetheless has a high descent death rate (probably because of high avalanche risk), whereas Lhotse is relatively big but has a low descent death rate (Figure 2). Nevertheless, the overall pattern is suggestive and consistent with physiological considerations: higher Himalayan peaks are deadlier, at least during descent from the summit.

Supplemental oxygen and death rate

In 1878 Paul Bert proposed using supplemental oxygen to reduce the physiological deterioration caused by hypoxia at altitude (1). Bert's suggestion was first implemented in the Himalaya early last century, and is still heavily used there on the highest peaks until this day. The use of supplemental oxygen has, however, always been controversial. For example, some climbers feel that supplemental oxygen use is unsporting (10,25). Even

so, supplemental oxygen does enhance performance at high altitude (15), and thus it might possible enhance survival as well.

We recently analyzed (7) a possible association between use of supplemental oxygen and death rates during descent from the summits of Everest and K2, the two highest peaks in the world. Our main data set included the years 1978, when the first ascents without supplemental oxygen were made on these peaks, through 1999. We found that death rates during descent were elevated for climbers who had not used supplemental oxygen, and the pattern was especially conspicuous on K2. In 2000 climbers were very successful on both mountains, and only one death occurred during descent (on Everest). To determine whether the pattern still held, we therefore reanalyzed the data, adding the data from 2000 (Figure 3). We used an exact logistic regression, with individual survival as the dependent variable, with supplemental oxygen use as a factor, and mountain as a stratum (7).

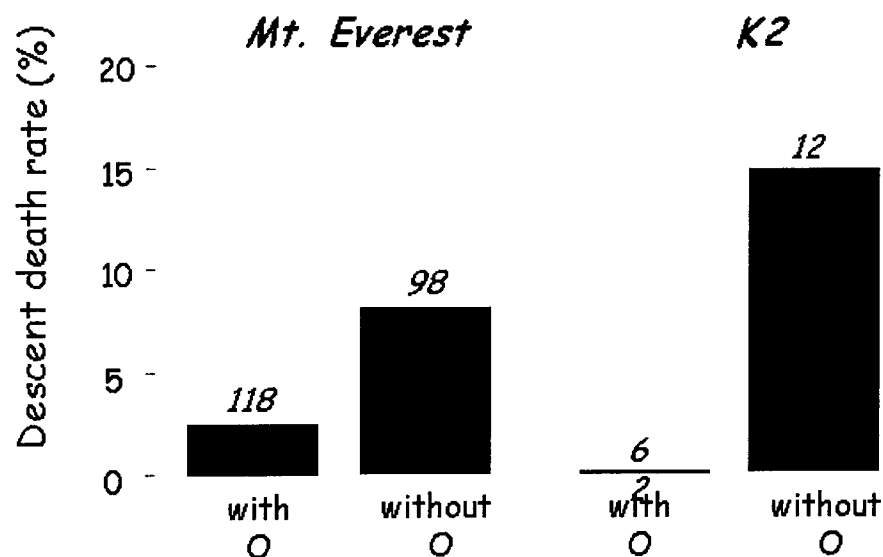


Figure 3. Death rates during descent from the summit of Everest and of K2 as a function of use of supplemental oxygen, with the number of individual summits indicated (through 2000).

Death rates during descent were lowered when the 2000 data were added to the original data set, but the percentage changes are small. Death rates during descent are still significantly higher ($P < 0.001$) for climbers who did

not use supplemental oxygen. This pattern remains significant even if one excludes deaths associated with two major storms on K2 in 1986 and 1995.

The statistical association between supplemental oxygen use and reduced death rate is strong, but does not necessarily imply cause and effect. Elsewhere we have outlined some alternative explanations of the patterns (4,7). For example, climbers who use supplemental oxygen use might survive better – not because of supplemental oxygen – but because they have better equipped high camps and Sherpa support in the event of a storm or medical problem (L. Reichardt, personal communication). Quantitatively evaluating such alternatives will require far more detailed data than are available at present.

We do note, however, that most of the deaths during descent on K2 and Everest occurred high on the mountain. Also, most of these deaths are from “falls” or “disappearances,” which probably implies a fall (otherwise most bodies would have been found). This pattern would be consistent with the idea that climbers descending from a high summit are often at their physiological edge, such that the use of supplemental oxygen could be a benefit in promoting survival.

How does convective heat loss change with altitude?

The above analyses focus on mountaineering statistics. But a full understanding of patterns of success and of death require an appreciation of the physical environment at high altitude. Barometric trends are now well understood (29), but our knowledge of temperature and wind at extreme elevation is still rudimentary. Yet many deaths in the Himalaya are associated with storms (9). So we are also beginning to study variation in physical environmental factors (especially temperature and wind), and how they impinge on organisms at high altitude.

A major problem for Himalayan mountaineers is convective heat loss, which influences wind chill and the resultant risk of hypothermia and frostbite (22). Although convective heat loss obviously increases with altitude, the relationship between altitude and convection is biophysically complex. Increased wind speeds and decreased air temperatures at altitude (16) will increase convective heat loss. However, air density declines dramatically with altitude (by ~ 60% from sea level to 9000 m) and will have the opposite effect on convection (C. Houston, personal communication). Thus, do equations using sea-level densities of air significantly overestimate heat loss (“wind chill”) at altitude? This evaluation requires a biophysical analysis of heat flux (22).

We have made an initial exploration of this issue. The model estimates the heat flux density for an exposed human face, assumed to be flat and parallel to the wind, have a diameter of 15 cm, and a fixed skin temperature

of 36°C (18)). Empirically derived relationships were found for input variables that vary with altitude or temperature or both (air pressure, specific heat capacity, thermal conductivity, and dynamic viscosity (3,12); and heat flux was calculated for various combinations of altitude, wind speeds, and air temperatures.

Figure 4 shows convective heat loss at different wind speeds and altitudes. At any given wind speed, predicted convective heat loss is (not surprisingly) much higher at 9000 m than at sea level, primarily because air temperature drops steeply with altitude ($\sim 6.5^\circ\text{C}/1000\text{ m}$). However, the counter impact of declining air density (" ρ ") is nonetheless strong. To show this, we plot the predicted heat loss for a air temperature appropriate for 9000 m, but with an air density appropriate for sea level (dashed line in Figure 4). This pattern may provide some consolation to mountaineers – the wind chill isn't as bad as it could be!

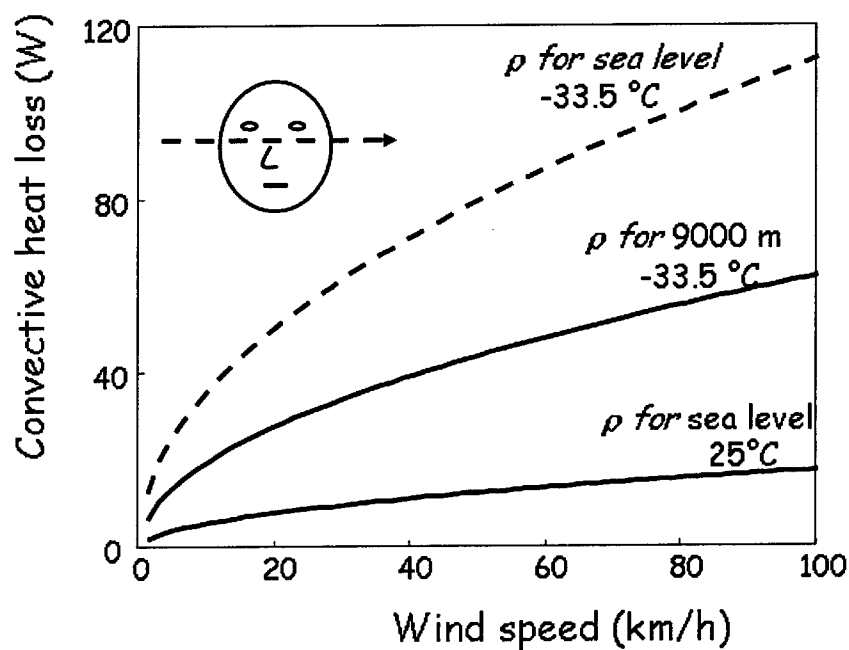


Figure 4. Predicted convective heat loss at different wind speeds, air temperatures, and air densities.

Weather and storms

The impact of storm on Himalayan mountaineers is well appreciated, at least in a subjective sense. However, no quantitative study has yet related

weather patterns to death or success rates. An obvious reason is the lack of standardized weather data for the mountains themselves.

An important innovation in this regard is the establishment of a high-altitude weather station by the Department of Applied Hydrobiology of the Italian National Research Council (23). The Pyramid station (5050 m) near Mt. Everest was established in 1990 at the confluence of the Lobuche and Khumbu Glaciers, just a few kilometers WSW of Mt. Everest. This station records air temperature, wind speed and direction, relative humidity, precipitation, and barometric pressure at 2-h intervals. Potentially such weather stations may be able to provide advance warning of approaching storms. Moreover, time-series analyses can potentially reveal unexpected patterns. For example, a recent analysis of data from a Tibetan station (4302 m) revealed a biennial cycle of winter precipitation (2). Such information could be extremely useful to climbers trying to decide when to schedule trips.

Long-term weather data will also provide information on the probability of storms. For example, was the infamous '96 storm on Everest a freak event, or do storms of similar magnitude occur there regularly? As the Pyramid data base grows, quantitative answers to such important questions will be possible.

CONCLUDING REMARKS

We have shown that quantitative analyses of mountaineering in the Himalaya often reveal conspicuous patterns. At a minimum level, those analyses can be useful in validating – or contradicting – conventional wisdom as to what is safe or dangerous. Moreover, those analyses can provide tests of predictions deduced from basic physiological data. In two cases presented here, analyses were consistent with those predictions. However, in both cases, we have had difficulty testing for possible confounding factors. Thus, the best we can do is to say that the data are consistent with expectations, but we are unlikely to assign rigorously cause and effect. Even so, the emergent patterns may provide valuable information to climbers themselves.

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Chapter 16

Weight loss at high altitude

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Abstract: Loss of appetite and weight are frequently observed at altitudes above 5000m. However, the pathophysiology behind changes in body composition at extreme altitude is still not fully understood. Proper acclimatization to altitude and high caloric intake minimizes, but can not completely prevent significant weight loss under the influence of hypobaric hypoxia. The discovery of leptin in 1994 has initiated a new research area investigating molecular networks that connect peripheral organs with the central nervous system to sense and regulate energy intake as well as energy expenditure. Since then, a whole microcosm of new hormones, neurotransmitters and receptors has been discovered and studied with respect to body weight control. Those agents include neuropeptide Y (NPY), agouti-related protein (AGRP), melanocortin receptors (MC-R), cocaine-amphetamine regulated transcript (CART), pro-opiomelanocortin (POMC), orexin A and B (hypocretins), melanin-concentrating hormone (MCH) and ghrelin (endogenous ligand of the growth hormone secretagogue receptor). This overview will introduce the current concepts of the molecular control of energy homeostasis and attempt to reexamine the effects of altitude on appetite and body composition in light of these concepts. An overview of studies on changes of appetite and body composition at high altitude will be followed by the presentation of recent data on changes of endocrine parameters at hypobaric hypoxia that could be involved in the pathophysiology of weight loss.

Key words: leptin, NPY, ghrelin, cachexia

INTRODUCTION

Weight loss and decreased appetite occur frequently at high altitude, with the extent depending on the duration of exposure and the altitude reached (1, 2, 5, 6, 8, 13, 16, 25-29, 41, 43, 51, 52, 53, 59, 60). However, the pathophysiological mechanisms behind weight reduction, changes in body composition and impaired appetite under the influence of hypobaric hypoxia are still unknown. Weight loss results from a marked difference between energy intake and energy expenditure, assuming that absorption of nutrients is not impaired. Proper acclimatisation to altitude and high caloric intake with a wide variety of nutrients can help to minimise but cannot completely prevent significant weight loss at high altitude (26) (3). What effect high altitude has on metabolic systems related to weight maintenance is still not fully understood. All three mechanisms contributing to total energy expenditure: physical activity, basal metabolism and adaptive thermogenesis, may be changed at high altitude and could therefore be partially responsible for an impaired energy balance in this extreme environment. This article will briefly review what is known about the regulation of energy balance at high altitude, considering recent new findings on the neuroendocrine regulation of energy homeostasis.

CHANGES IN BODY COMPOSITION AT ALTITUDE

Changes in body composition caused by exposure to high altitude have been studied in numerous studies in search of pathophysiological explanations. The degree of weight loss varies depending on the altitude achieved, and length of stay there, and has been as extreme as 8.9% of body weight at outset after a 62-day expedition to 8047m (16). In most studies, the majority of the lost body weight is attributable to loss of fat mass (5) (18) (8) (26) (27) (28) (52) (53) (1) (40) (3) although decreases in muscle mass, accompanied by a negative nitrogen balance have been described as well (18) (43). One study has suggested however, that although under 5000m, 70% of the weight loss is fat loss, over 5000m only 27.4% of weight loss is secondary to loss of fat (5). Effects were not consistent over all groups however, as Sherpas who arrived in Base Camp with half as much body fat, maintained their weight during residence above 5400m. Although most studies report that most of the body weight lost at high altitude consists of body fat, many experienced mountain climbers anecdotally report severe loss of muscle mass during expeditions. One possible explanation for this discrepancy could be differences between the populations chosen for studies with simulated high altitude (normal volunteers) and populations of field studies (highly trained mountaineers). We hypothesize that exposure to

hypobaric hypoxia might initially and principally cause loss of fat mass in normal volunteers, but if fat stores are reduced or nearly non-existent due to extensive precedent physical exercise, exposure to high altitude might also cause loss of lean (muscle) mass.

DIETARY CHANGES AT ALTITUDE

To determine whether the hypoxia which occurs during decompression, causes weight loss, a study was conducted by the US Army Research Institute of Environmental Medicine. Six men, provided with a palatable ad libitum diet, were studied during progressive decompression to 240 Torr over 40 days in a hypobaric chamber where hypoxia was the major environmental variable. Caloric intake decreased 43.0% from 3,136 to 1,789 kcal/day (P less than 0.001). Over the 40 days of the study, the subjects lost 7.4 \pm 2.2 (SD) kg and 1.6% (2.5 kg) of the total body weight as fat. Computerized tomographic scans indicated that most of the weight loss was derived from fat-free weight. The authors concluded that hypoxia can be sufficient cause for the weight loss and decreased food consumption reported by mountain expeditions at high altitude (43). Efforts to eliminate weight loss at high altitude have included increasing energy intake, as described above. Researches have also attempted to ameliorate the effects of altitude with acetazolamide (Az). In a placebo-controlled trial, Bradwell and coworkers examined exercise performance and muscle mass in 21 acclimatized subjects at an altitude of 4846m. Although weight loss was less and exercise performance was better in the Az treated group (n=11), altitude effects were not completely prevented (6). It appears, therefore, that hypoxia during high mountain expeditions induces weight loss by hypophagia, and possibly malabsorption and increased metabolic rate. A brilliant study by Westerterp-Platenga and coworkers examined changes in appetite during simulated exposure to high altitude, that way excluding confounding factors that usually occur at high altitude in addition to hypobaric hypoxia such as cold, psychological stress, limited availability of palatable food and overexertion (57) (56). Eight healthy men were studied during a simulated ascent on Mount Everest (7 days baseline measurements, 7 days field acclimatization (Mont Blanc, 4350 m), 31 days simulated ascent Mount Everest (8848 m)). Significant weight loss (5 \pm 2 kg, $p < 0.05$) was observed in the studied population, even though environmental factors other than hypobaric hypoxia had been excluded by use of the hypobaric chamber (57) (56). The negative energy balance causing weight loss in this experiment was mainly due to reduced food intake (more than 50% reduction), since energy expenditure tended to be decreased at simulated high altitude (57) (56). Reduction in food intake during exposure to high altitude has been

published before (5) (18) (52) (13) (19) and seems to occur at least in part independent of other symptoms of acute mountain sickness (AMS) (56, 57). "Decreased appetite preventing from reaction to hunger" at high altitude has been termed "appetite profile uncoupling" and is regarded as the main reason for reduced food intake at high altitude (56) (57) (51).

In addition to a total reduction of food intake, a change in feeding pattern from a "gorging"-style (less frequent, bigger meals) to a "nibbling"-style (more frequent smaller meals) was documented at high altitude (53). This effect was paralleled by a relative increase in carbohydrate intake (55). Still, the lost body weight was mainly due to increased fat oxidation and loss of fat mass (55). In summary, decreased caloric intake (18); (40) and based on that, a negative energy balance (52, 53) have been noted at high altitude, and are considered the major contributors to weight loss. The importance of appropriate quality and composition of nutrients during sojourns at high altitude has been repeatedly emphasized in the literature (7, 8, 17, 18, 20, 23, 25, 26, 28, 43, 53, 54, 59, 60). However, efforts to overcome hypoxia induced anorexia with a wider variety or better composition of food does not fully prevent weight loss (7, 8) (3). Malabsorption of fat has also been noted, but is not confirmed in all studies (9) (27).

Leptin and the Control of Energy Balance

A critical brain region for the regulation of homeostatic processes is the hypothalamus: it senses neural, endocrine and metabolic signals, integrates these inputs and engages distinct effector pathways, resulting in behavioural, autonomic and endocrine responses. Body weight remains relatively stable under physiological conditions, with either weight loss or gain producing concerted changes in energy intake and expenditure that counter the initial perturbation (44) (46). This indicates that energy balance may be controlled by a feedback loop involving a so-called "set-point", in order to maintain constancy of total body energy stores. It has been proposed that signals reflecting nutritional state are sensed by hypothalamic neurons, where, in turn, food intake and energy expenditure are modulated by a multi-levelled central network that influences endocrine, behavioural and autonomic effector mechanisms. In addition to the hypothalamus, central control of appetite and energy balance clearly involves widely distributed neural systems in the brainstem, cerebral cortex and olfactory areas, amongst others (46) (11) (15) (14) (42) (44) (24). The demonstration that hypothalamic lesions can cause hyperphagia, decreased energy expenditure and obesity as well as hypophagia and cachexia has led to the homeostatic model of body weight regulation (46). Until recently, the key players in this system had not been identified, but the discovery of leptin and other hormones and neuropeptides within the last decade has shed considerable light on what was

once a black box (12, 14, 15, 22, 24, 30, 32, 33, 44, 45, 47, 58). The discovery of a new microcosm of molecules controlling energy homeostasis begins with the ob/ob mouse, a mouse strain that was first described in the Jackson Laboratories. A recessive gene mutation in the ob/ob mouse produces a phenotype characterised by the behavioural trait of hyperphagia and the morphological trait of obesity, resulting in sterile adult mice with 50% body fat content. Similar phenotypic features are seen in db/db mice, but they also suffer from diabetes. Coleman, from the Jackson Laboratories (10) conducted the groundbreaking parabiosis (inter-individual cross-circulation) studies in which the ob/ob mice, once exposed to the circulation of db/db mice decreased their food intake and body weight. In contrast, their db/db pairmates, although exposed to the circulation of ob/ob mice, continued to increase their food intake and weight. Coleman concluded, that ob/ob mice fail to produce a circulating factor important in appetite control, that their brains can respond to, whereas db/db-mice make the circulating factor in abundance, but their brains are unable to respond to it. After extensive genetic studies in ob/ob mice, Zhang and co-workers (61) reported that they had identified the gene responsible for obesity in these mice, and it encoded a 146 amino-acid protein. Because this protein, the obese gene product, caused a reduction in food intake (as well as an increase in metabolic energy expenditure) it has subsequently been called leptin from the greek "leptos" for "thin". It is now known that leptin is secreted by adipocytes and, in lesser amounts, also by the placenta, muscle and stomach (45) (47) (15) (46).

The findings that the administration of recombinant leptin led to impressive weight loss in rodents and that leptin also signals nutritional status to the brain and modulates the function of other physiological systems showed that leptin obviously plays an important role in the regulation of energy homeostasis. Effects of leptin on pubertal development, fertility, hematopoiesis, immune function and angiogenesis have also been described.

The initial hypothesis, that human obesity is also explained by leptin deficiency has, however been proven wrong. In general, obese individuals have elevated leptin levels that correlate closely with body fat mass (12). This is not to suggest that leptin deficiency never results in obesity in man. In fact, O'Rahilly and co-workers have described two young cousins with a genetic leptin deficiency, marked hyperphagia and extreme obesity (33). Although a rare cause of obesity, these patients have confirmed the essential role of leptin regulation of energy homeostasis in humans. In spite of the effects of leptin in rodents on food intake and metabolic energy expenditure hopes for the use of leptin in the treatment of human obesity (other than in the rare leptin deficient patients noted above) have not been realized. (22) (30).

Leptin at Altitude

To determine if leptin is involved in the pathogenesis of altitude induced weight loss and anorexia, we investigated the effect of exposure to high altitude on circulating leptin levels in men (51). Plasma leptin concentration significantly increased in 20 male mountaineers after active ascent to 4559 m (Capanna Margherita, Switzerland). This effect was not reversible after treatment with oxygen-enriched air and appeared to be more pronounced in subjects with loss of appetite, than in those without loss of appetite (standardized questionnaires). In a second study, in 18 volunteers, 70% of the individuals studied developed loss of appetite, and 10 developed AMS after passive transport to high altitude (4559 m). In individuals with loss of appetite, but not in those without loss of appetite, circulating leptin levels were increased (51).

Ten individuals who developed AMS also showed significantly increased leptin levels when compared to their baseline levels at normoxia, even though effects due to change in plasma volume had been excluded and mean body mass indices were not significantly different between any of the analysed subgroups. Further evidence for a regulation of leptin secretion by hypoxia has been suggested by the work of Mise and collaborators demonstrating that placenta derived leptin is increasingly secreted with placental hypoxia, and that leptin expression in cells cultured under hypoxic conditions was higher than under standard conditions (32).

Based on these findings which show an increase in leptin secretion under the influence of hypoxia, and the known fact that hypoxia can trigger anorexia and weight loss, we hypothesize that leptin may be directly involved in the induction of anorexia and weight loss at high altitude.

The Hypothalamic-Pituitary Axes at Altitude

Multiple clinical and experimental studies have demonstrated changes in endocrine parameters at high altitude. However, the mechanisms mediating these changes are by far not clearly understood. Because of their effect on energy homeostasis, thyroid hormones have been considered a possible contributor to weight loss. At high altitude, TSH secretion from the pituitary gland appeared to be enhanced and total and free thyroxine were found to be elevated, but peripheral conversion from T_4 to the active form of thyroid hormone, T_3 was impaired. Correlation of these changes with marked weight loss was not found (34) leaving their importance in the weight loss phenomenon in question.

Another important system for the regulation of body composition is the somatotrophic axis including growth hormone (GH), growth-hormone-releasing hormone (GHRH), insulin-like growth factor-I (IGF-I) and IGF-binding proteins. Increased hGH response to administration of the GH

releasing stimulant GHRH, in healthy volunteers at high altitude has been demonstrated (39) (38), but levels of the effector protein IGF-I remained unchanged. An increase in IGF-binding proteins at altitude has also been recently reported, but the relation of these to weight loss remains unclear (49).

The first work examining the role of the gonadotropic axis, including LH, FSH, estrogens and androgens, in weight loss at high altitude was published by Martin and coworkers in 1977. They showed that weight loss in castrated rats at simulated altitude of 6000 m is not reversible by administration of testosterone or estradiol (31). In most of the studies analysing the function of the gonadotropic axis during hypoxia, no significant changes in LH, FSH, testosterone or estradiol-levels with hypoxia were found compared to normoxic conditions in men (4).

Studies on the effects of hypoxic conditions at high altitude on the CRH-ACTH-Cortisol axis have demonstrated increases in serum cortisol levels with a concomitant loss of the typical diurnal rhythm of ACTH and cortisol secretion (48). This increase seems to be suppressible by administration of exogenous corticosteroids (37) and its relationship to weight changes is unknown. Changes in endocrine function at high altitude are described frequently, but results and conclusions are often quite contradictory. An endocrine link between the changes induced by hypoxia, and energy homeostasis or weight loss is still missing. Based on published data observing the endocrine changes in the traditional hypothalamic-pituitary-periphery-axis one cannot conclude if or how the phenomenon of weight loss at high altitude is hormonally mediated.

Cytokines and Cholecystokinin at Altitude

It is unlikely, however, that the significant, but no means spectacular increase in circulating leptin levels alone causes the substantial physiological changes leading to weight loss at high altitude. Various cytokines, for example, are also able to induce anorexia (35) (36). Significant increases in circulating interleukin-6, TNF- α and C-reactive protein in 8 healthy individuals after 2 days at 4559 m have been shown (21). Increased cytokine levels associated with clinical symptoms were also demonstrated in a study of 10 male subjects at an altitude of 3600 m (Jungfrauoch, Switzerland) (21). Another agent that seems to be involved in the pathophysiology of hypoxia induced weight loss is the satiety inducing peptide hormone cholecystokinin (CCK) (3) (2). Bailey and co-workers were the first to demonstrate, in very carefully conducted studies, an increase in circulating human CCK-levels at high altitude. This increase appeared to be more pronounced in subjects that suffered from AMS (3). One more interesting molecule in that respect could be the new gastric hormone ghrelin, an

endogenous ligand of the growth hormone secretagogue receptor, that is known to regulate growth hormone secretion at pituitary cells and has recently been shown to function as a peripheral opponent to leptin (50).

SUMMARY

As understood today, leptin, ghrelin, and many other peripheral hormones and signalling molecules involved in the regulation of energy balance, modulate a system of central neuropeptides and their receptors. Although expanding knowledge of the action and pathways of essential neuropeptides such as neuropeptide Y (NPY), proopiomelanocortin (POMC) or Agouti-related protein (AGRP) is now available, their systemic interplay is not fully understood and the clinical application of compounds that modulate this system exists only in theory. A detailed investigation of hypoxia-induced changes in the neuroendocrine network involving leptin, ghrelin, CCK and cytokines as well as hypothalamic neuropeptides is necessary to solve the mystery of weight loss at high altitude. Once more details on the pathogenesis of anorexia at high altitude are known, therapeutic strategies to prevent uncontrollable weight loss in mountaineers during high altitude expeditions can be mapped out.

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Chapter 17

The heme oxygenase system and cellular defense mechanisms

Do HO-1 and HO-2 have different functions?

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Abstract: Heme oxygenase isozymes, HO-1, HO-2 and HO-3, are HSP32 protein cognates, with a known function of catalyzing the isomer specific oxidation of the heme molecule, including that of NO synthase. Unknown until recent years was that the system is a central component of the cellular defense mechanisms; this can be attributed to a combination of many factors. In biological systems HO activity is responsible for production of equimolar amounts of CO, biliverdin and free Fe. The serine/threonine kinase, biliverdin reductase, catalyzes reduction of biliverdin to bilirubin. Bilirubin is a potent antioxidant and CO is a signal molecule. Although both active HO isozymes catalyze the same reaction, HO-1 and HO-2 may function in a rather distinct fashion in protection against tissue injury. HO-1 is the stress responsive cognate that is rapidly induced by free and stable radicals as well as by hypoxia. Supra induction of HO-1 completely protects ischemic kidney against tissue pathology. This involves rapid inactivation of the pro-oxidant heme of denatured hemoproteins and converting it to bilirubin and CO. In the case of severe tissue injury, such as compression injury, HO-1 is induced and colocalizes with cGMP and pro-apoptotic oncogenes. HO-2, which is the constitutive form, in addition to maintaining cell heme homeostasis, inactivates NO derived radicals. The isozyme binds the free radical at its "heme regulatory motifs" and is "suicide" inactivated at the protein and transcript levels. Data are shown that provide evidence for role of the HO system in the cellular defense mechanism against free radical-mediated tissue damage, and are consistent with the forwarded concept that HO isozymes have common, as well as distinct, roles in cellular defense mechanisms.

Key words: bile pigments, carbon monoxide formation, heme oxygenase, kidney ischemia/reperfusion, oxidative stress, spinal cord injury

INTRODUCTION

The most effective mechanism for biotransformation and disposal of heme moiety of denatured hemoproteins, including hemoglobin, is the heme oxygenase (HO) system (reviewed in 31). The enzyme system oxidatively cleaves heme to biliverdin, forms CO, and releases the chelated Fe. Biliverdin is subsequently reduces to bilirubin by the serine/threonine kinase, biliverdin reductase (67). The heme degradation pathway is schematically shown in Figure 1. We identified the HO system in the 1970's as a distinct microsomal entity and characterized it as a stress-inducible enzyme (35). Later, a second form of the enzyme, we called HO-2 was discovered (41,79). More recently we identified a third form, HO-3 (47), which has negligible heme degrading activity. The two fully characterized forms, HO-1 and HO-2, are both phosphoproteins (67), but are dissimilar in protein structure, tissue distribution and gene organization and regulation. (7,17,18,47,50,66,66). HO-1, also known as the heat shock/stress protein (HSP32), is an immediate early gene (18) and is exquisitely sensitive to a multitude of stimuli that cause oxidative stress (reviewed in 30,31) including hypoxia (4). In contrast, the list of inducers of HO-2 is limited and include developmental factors, adrenal glucocorticoids, opiates and possibly nitric oxide (9,27,28,33,64,75).

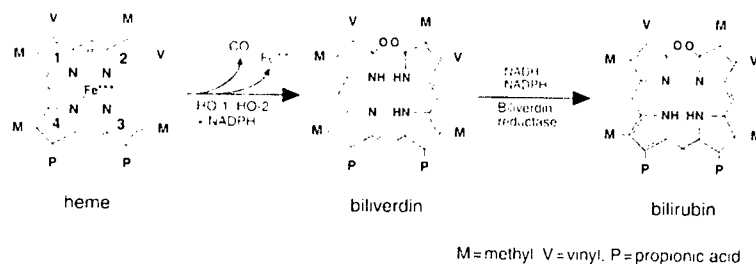


Figure 1. Schematic presentation of heme degradation pathway.

It is well documented that the catalytic activity of the HO system plays a crucial role in maintaining cellular heme homeostasis (reviewed in 31). In fact for nearly 2 decades this was the only function associated with the enzyme system. In recent years other functions have been ascribed to the system. Our laboratory, along with others, has implicated CO in signaling mechanisms, in the brain and other organs, and in activation of soluble guanylate cyclase and generation of cGMP (11, 19, 25, 32, 34, 37, 42, 43, 48, 49, 58, 61, 62, 63, 65, 70, 74, 80, 85, 90). cGMP is essential for vasodilatation responses of the microcirculation (21). Gaseous signal molecules, NO/NO derivatives and CO, can modulate the signaling cascade caused by hypoxia (29).

Iron released by HO activity regulates several genes, the iron storage protein, ferritin; the iron transport protein, transferrin; NO synthase; and HO-1 being among them (36,60,81,86). Redox available iron is a potent catalyst for oxygen radical formation and lipid peroxidation (2). Iron sequestered into ferritin complex is not catalytically active, hence by promoting increased synthesis of ferritin, HO activity limits the iron available for reactivity with oxygen (8,60). In addition, the reduction product of biliverdin, bilirubin, formed by activity of biliverdin reductase (20,26,29), scavenges oxygen free radicals and, in this respect, is as effective as the potent antioxidant, tocopherol (52,71,72). In fact, it has been documented that in man, higher serum levels of bilirubin is associated with decreased risk for early familial coronary artery disease (24). In addition, biliverdin and bilirubin are known to inhibit inflammatory response (reviewed in 87). Bilirubin has been shown to inhibit interleukin-2 (IL-2)-stimulated killing activity of lymphocytes (51), IL-2 production and antibody-dependent and independent cell-mediated cytotoxicity (89). In addition, CO may afford protection by inhibiting platelet aggregation (5), and as noted above, by stimulating generation of cGMP and promoting vasorelaxation (11,74).

Several studies have shown that upregulation of HO-1, and the concomitant increased production of active products, protects organs against ischemia/reperfusion injury (1,38,40,53,55). In fact, the utility of upregulation of HO-1 activity has been explored for prolongation of transplanted organs (12,56,71,89). It has been documented that an increased HO-1 gene expression prolongs transplanted organ half life, whereas an HO-1 deficient heart is vigorously rejected (56,71). And, granule neurons isolated from HO-1 Tg mice show increased resistance to oxidative stress caused by exposure to glutamate and H_2O_2 (6). Although both active forms of HO catalyze the same reaction with respect to heme degradation, it is reasonable to suspect that differences that exist in the primary structure of HO-1 and HO-2 and regulation of their gene expression, may lend to their additional and differential function in defense mechanisms. HO-2 (but not HO-1) is among a select group of six proteins that have a heme binding motif known as the "heme regulatory motif" (HRM) and has been suggested to function as a "sink" for NO and gaseous heme ligands (9,34). The investigation described here are consistent with possibility of differential role of the HO isozymes in cell/tissue defense against oxidative stress.

MATERIALS AND METHODS

Materials

Oligo (dT) cellulose, DNase I, Salmon testes DNA, N-tert-Butyl α -phenyl nitron (PBN), and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for mRNA isolation and HO isozyme cloning were purchased from USB Corporation (Cleveland, OH). Reagents for immunohistochemical studies were products of Organon Teknika Corporation (Westchester, PA), Chemicon Int. (Temecula, CA), Zymed (San Francisco, CA), or Vector Labs (Burlingame, CA). Male Sprague-Dawley rats (290-370 g) were purchased from Harlan Industries (Madison, WI) mice were DNX strain. Purified E.coli expressed HO proteins: rat HO-1, HO-2 and HO-2 mutant proteins were prepared as previously described (9,45). Rat HO-2 in which cysteine 264 and cysteine 281 were replaced by alanine residues (Cys²⁶⁴/Cys²⁸¹→Ala/Ala), and referred to as HO-2 mut was generated by site-directed mutagenesis (45). All chemicals were of highest purity commercially available.

Experimental models and tissue preparation

All animal treatments were performed in strict accordance with NIH guide for the care and use of Laboratory Animals as approved by the University Committee for Animal Resources. To induce renal ischemia, rats were anesthetized with pentobarbital (40 mg/kg; ip), and as described before (40), 30 min prior to induction of ischemia; were treated with PBN (100 mg/kg, ip) in 100 μ l of DMSO. Control rats received 100 μ l of DMSO. Rats were also subjected to renal ischemia or sham-operation, without PBN pretreatment. Renal ischemia was induced by means of occlusion of both renal arteries for 30 min as described before (38). At time points indicated in appropriate figure legends rats were killed and kidneys and/or heart were processed. A clip compression injury of the spinal cord was performed as described earlier (54), which employs minor modifications to the procedure described for the rat (83). The time of clip compression was selected to be 30 min.

Northern blot analysis

poly(A)⁺ RNA isolated from rat kidney or heart by oligo (dT)-cellulose chromatography and the formaldehyde denatured RNA was fractionated on 1.2% (w/v) agarose gel and transferred to Nytran membrane. Prehybridization and hybridization of the membranes with the appropriate [³²P]-labeled cDNA were performed essentially as described before

(18,19,75). Full length HO-2 cDNA (66) and a 500 bp fragment of HO-1 (68) were used as probes. The membranes were exposed at -70°C to Kodak X-OMAT film with intensifying screens and autoradiographs were quantified using BioRad model GS-700 imaging densitometer.

Immunocytochemical protocols

Procedures described in detail previously were used (15,40,54). Briefly, 24h after induction of reperfusion rats were given an overdose of pentobarbital (100 mg/kg; ip) and perfused transcardially first with heparinized saline, then by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) and post fixated in 4% PFA at $4-6^{\circ}\text{C}$. Kidneys or the spinal cord were cryoprotected in 30% ethylene glycol and 20% sucrose in phosphate buffer (2-3 days) at 4°C . Kidneys were cut in 25 μm thick cross sections. The spinal cord was cut longitudinally in 35 μm thick sections. Staining of tissue from control and treated animals was carried out under identical conditions using same reagents and solutions.

HO-1 was detected as before (40) using 3B8C8 monoclonal antibody developed in collaboration with StressGen (Vancouver, Canada) at 1/1,000 dilution. Biotinylated secondary antibody and avidin-biotin reagents were used according to manufacturer's recommendations for peroxidase detection (Vectastain Elite mouse, IgG kit, Vector Labs). Rabbit anti-ferritin and anti-transferrin antibodies (Zymed) were used at 1/250 and 1/100 dilutions, respectively. Vimentin was detected using a monoclonal IgM anti-vimentin antibody (Chemicon Int.) in dilution of 1/200. Peroxidase detection (Vectastain Elite, rabbit IgG kit), with 3', 3'-diaminobenzidine (DAB) as the chromogen were utilized. cGMP was visualized using an antisheep monoclonal antibody as provided generously by Professor deVente and described before by deVente and colleagues (77). Sections were incubated overnight at $4-6^{\circ}\text{C}$ with anti-cyclic GMP antibody in a dilution of 1:4000 and then processed with a secondary antibody and avidin biotin reagent (Vectostain Elite sheep IgG kit #PK-6106). Vector SG substrate kit was used as the chromagen. For double immunolabeling studies, HO-1 antibody was used first, followed by anti-cGMP antibody staining.

Anti-mouse p53 and anti-bcl₂ monoclonal IgG (Santa Cruz Biotechnologies) were used in 1/500 dilutions. Anti-Traill, anti-Rip and anti-Bax affinity-purified goat polyclonal antibodies of mouse origin, were also obtained from Santa Cruz Biotechnologies and used at 1/100 dilution. DAB was always used as a primary chromogen, while SG was used as the secondary chromogen in all double labeling studies. In all double immunostaining experiments HO-1 antibody was used first.

Histochemical detection of iron and lipid peroxidation

Iron was detected as described by Smith et al. (69). Lipid peroxidation at tissue level was assessed as before (40) using free floating specimens as described by Pompella et al. (59), which utilizes Schiff's reagent (filtered pararosaniline base: thionyl chloride).

RESULTS

Response of HO-1 and HO-2 transcripts to ischemia/reperfusion in the kidney and heart

The effect of PBN pretreatment on HO-1 transcript levels in kidney, subjected to 30 min ischemia followed by 24 h reperfusion, was examined by Northern blot analysis. The levels of mRNA were normalized to that of GADPH mRNA for calculation of the relative levels of transcripts. As shown in Figure 2, at this time point the levels of HO-1 ~1.8 kb transcript were increased by approximately 7-fold in ischemic/reperfused kidney, when compared with those of sham-operated animals. Pretreatment of rats with PBN 30 min before ischemia/reperfusion markedly augmented induction of HO-1 mRNA, when compared with sham operated rats receiving PBN alone. At this time, an astonishing increase of nearly 40-fold in HO-1 mRNA levels was detected. It is notable that administration of PBN alone to sham-operated animals did not significantly increase the HO-1 mRNA levels.

Next the effect of ischemia of reperfusion on HO-2 mRNA levels at 24h was examined. Also, the response of HO-1 mRNA levels at 4h after ischemia/reperfusion was compared with that of HO-2 at this time point. The results are shown in Figure 3. As noted in panel a, at 4 h after experimental manipulations, HO-1 mRNA levels were already increased by nearly 2.5 fold; this was in contrast to the response of HO-2 mRNA, which was pronouncedly reduced at this time point (panels b). When examined at 24 h decrease in HO-2 mRNA levels persisted (panel c), the decrease was detected in both 1.3 kb and 1.9 kb transcripts. Pretreatment of rats with PBN 30 min before the onset of ischemia/reperfusion blocked the decrease in HO-2 mRNA levels (panel d).

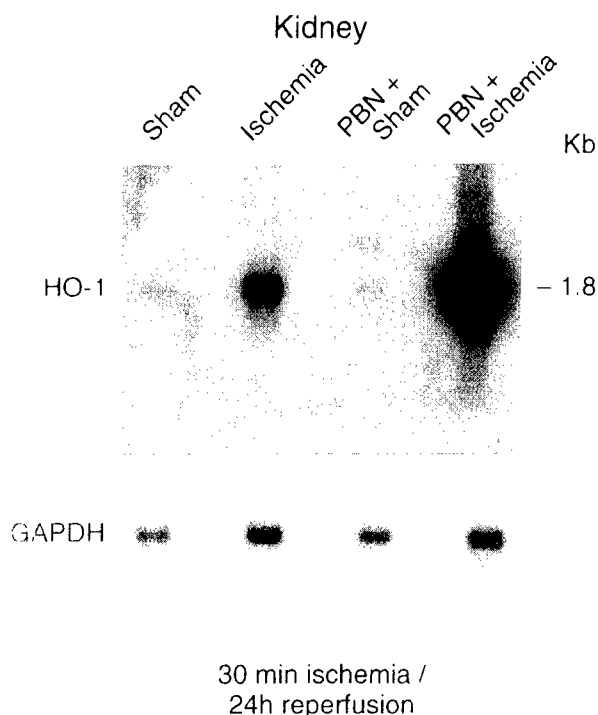


Figure 2. N-t-Butyl- α -phenylnitron potentiates induction of HO-1 mRNA in ischemic/reperfused kidney. Rats were treated with 100mg/kg (ip) PBN or vehicle (DMSO) 30 min before being subjected to bilateral ischemia. After 30 min of ischemia, reperfusion was resumed and rats were killed 24 h later. Poly(A)⁺ mRNA was isolated from kidneys and used for Northern blot analysis. The blot was probed first with ³²P-labeled HO-1 cDNA and subsequently with GAPDH, which was used as the loading control. Each lane contained 4 μ g of RNA.

Two possible mechanisms may be considered to explain the HO-1 suprainduction phenomenon. The first possibility would consider activity of stable radicals formed through interaction of PBN with free radicals formed in the compromised organ in HO-1 gene regulation. According to this possibility, stable free radicals with a prolonged half life would be more effective in modulating gene expression by the virtue of their prolonged presence in the cell. The second possible mechanism for the suprainduction could involve the removal of cytotoxic free radical and reducing their bioavailability for direct interaction with cellular components, including gene transcription machinery. The decrease in HO-2 mRNA levels and protection offered by PBN support this concept and may reflect the susceptibility of HO-2 transcripts to free radicals. Reactive oxygen species formed in ischemic/reperfused organs can cause nucleotide strand breaks. Therefore, by trapping the powerful oxidizing radical species the rate of decay in HO isozymes' mRNA could decrease. This possibility is consistent

with the sustained remarkable elevation of HO-1 mRNA levels in PBN pretreated rats. Production of nitric oxide is increased in ischemic/reperfused tissue and oxygen radicals upon interaction with nitric oxide form highly reactive and toxic derivatives, e.g. peroxynitrite (3). The possibility also exists that nitric oxide itself is directly scavenged by the spin trap agent subsequent to its formation.

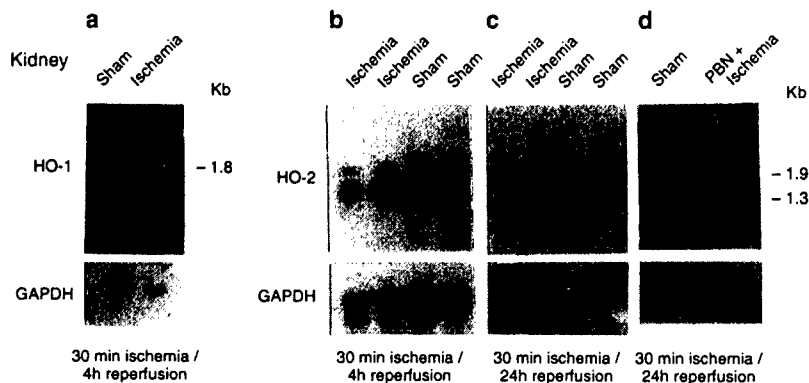


Figure 3. Ischemia/reperfusion decreases HO-2 transcript levels and N-t-butyl- α -phenylnitron pretreatment blocks decrease in HO-2 mRNA levels in kidney after ischemia/reperfusion. Rats were treated as described in the legend to Figure 1 and subjected to bilateral ischemia (30 min) followed by reperfusion for 4 or 24 h. Poly(A)⁺ mRNA was isolated from the organ and used for Northern blot analysis as described in Materials and Methods. For comparison, the response of HO-1 mRNA when measured 4h after ischemia/reperfusion also is shown. Panel a) HO-1 mRNA blot was probed first with a ³²P-labeled HO-2 cDNA followed by GAPDH. Panels b-d blots were probed with ³²P-labeled HO-2 cDNA followed by GAPDH. Each lane contained 4 μ g of poly(A)⁺ RNA.

The response to 30 min renal ischemia followed by 24 h reperfusion of HO-1 and HO-2 mRNAs in the heart is shown in Figure 4. When normalized to GAPDH signal, as noted, renal ischemia caused a significant increase in heart HO-1 mRNA levels over the sham-operated animals. The dramatic reduction at this time in heart HO-2 transcripts, particularly the 1.3 kb size is denoted. It appears that HO-2 mRNA is susceptible, independent of the organ, to factors which, based on the PBN pretreatment experiments with the kidney, include free radicals. Finding that pretreatment of rats with PBN prior to ischemia/reperfusion injury protects against the decrease and in fact leads to somewhat of an increase in the 1.9 kb transcript size is consistent with involvement of free radicals in decrease in HO-2 mRNA levels. Next we examined whether HO-2 protein is also vulnerable to free radicals, in this case NO radicals.

Data presented in Table 1 and Figure 5 support a plausible function for heme bound to the regulatory motifs of HO-2, with the core of Cys²⁶⁴-Pro²⁶⁵ and Cys²⁸¹-Pro²⁸², as a binding site for heme ligands (CO, O₂, NO); and, in the case of NO function as a "sink" for, and a modulator of cellular NO

levels and hence, influencing the course of NO-mediated reactions. In this process HO-2 is compromised. Heme iron is a target for the NO radical (3). The functional sites of proteins are also targets for NO donors and their reactive products. NO compounds, at physiological pH range, have high affinity for sulfhydryl and amine functional groups and form S-nitrosothiols and N-nitrosamines, respectively (88). Rat HO-2, in addition to 2 cysteines of HRMs, has a third cysteine, Cys¹⁴³ (66); human HO-2 has only 2 cysteines, both associated with HRMs (44) and HO-1 has no cysteine residues (68). The findings presented in Table 1 show that HO-1 activity and that of mutated HO-2 protein were essentially refractory to NO donors. In mutated HO-2, Cys²⁶⁴ and Cys²⁸¹ were changed to alanine. Based on the findings, it would appear that nitrosylation of reactive functional groups, including that of amino groups should they occur, does not have consequences to oxidation of heme by HO isozymes. Thus, it is reasonable to conclude that the basis for NO donor inhibition of wild type HO-2 resides in the interactions involving heme bound to its HRMs.

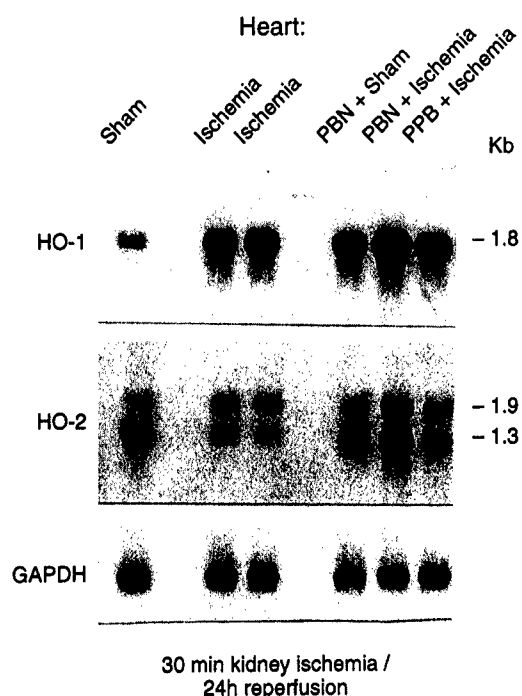


Figure 4. N-T-butyl- α -phenylnitron pretreatment increases HO-1 mRNA in the heart and prevents decrease in HO-2 mRNA levels after ischemia/reperfusion of the kidneys. Rats given DMSO or pretreated with PBN (100mg/kg, ip) were subjected to ischemia (30 min) and sacrificed 24 h after reperfusion. Heart was removed and used for Northern blot analysis. As described in the text. The blot was probed sequentially with HO-1, HO-2 and GAPDH. Each lane contained 4 μ g poly(A)⁺ mRNA.

Spectral analysis of HO-2: heme binding

As shown in Figure 5a interaction between NO species with heme bound to HO-2 HRMs occurs as indicated by change in the Soret region absorption spectrum of purified wild-type HO-2. As noted, S-nitroglutathione (GSNO), sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and 3-morpholiniosydnonimine (SIN-1) cause a shift to a higher wavelength (405→419 nm) of the Soret band. The shift in the Soret absorption band is likely caused by NO/NO derivatives displacing the labile sixth coordinate of heme iron; the proximal thiolate ligand involving cysteine would not be available for interaction with NO. Clearly heme bound to HO-2 is NO/NO derivative interactive, since HO-2 mut does not display heme absorption spectrum. Further data in support of HO-2 HRM high affinity interaction with heme is provided in Figure 5b and c. For these experiments, interaction of 10 residue long HO-2-based peptides were used and competition with cyanide, a heme ligand, was analyzed.

Table 1. Selective inhibition of HO-2-mediated heme oxidation activity by NO donors.

NO donor	Enzyme (% activity)		
	Wild type HO-2	HO-2- mutant	HO-1
None	100	100	100
SNAP	77	97	100
SIN-1	73	96	100
SNP	42	93	99

Purified *E. coli* expression preparations (1 μ M) or rat wild-type HO-2, HO-2 mutant or HO-1 were incubated with 100 μ M final concentration of the indicated NO donors for 1 h at 25°C, in the dark. Exchange into Tris-HCl buffer (pH 7.4) (3x) was used to remove excess NO donors before measuring heme degradation activity of the preparations. Activity of each protein preparation in the absence of NO donor was defined at 100% and was 2800 ± 100 , 1882 ± 124 and 2454 ± 136 nmol bilirubin formed/mg protein/h for wild-type HO-2, HO-2 mutant and HO-1, respectively.

Incremental additions of KCN to the heme:peptide solution split the single absorption band into two bands at 369 nm and 422 nm. As KCN concentration increased the 422 nm peak increased. Additions were continued until absorption was not affected. As indicated by the apparent isosbestic point, there is a direct conversion of one species, i.e heme iron-cysteine to another, i.e heme iron-CN with both peptides. The spectrum of heme-CN at the completion of titration was obtained at a remarkably great excess of KCN; 25 mM KCN for Cys²⁶⁴-Pro²⁶⁵ containing peptide and 15 mM KCN for Cys²⁸¹-Pro²⁸² containing peptide. This amounted to 6000-fold excess of KCN to Cys²⁶⁴-Pro²⁶⁵ peptide, and a nearly 4000-fold excess to

Cys²⁸¹-Pro²⁸² peptide. As a quantitative measure of the relative affinity of the peptides and CN for heme, the equilibrium constants for the KCN-mediated dissociation of the heme-peptide complexes measured and were 6.6×10^{-11} and 7.9×10^{-11} for HO-2 based peptide containing Cys²⁶⁴-Pro²⁶⁵ and Cys²⁸¹-Pro²⁸², respectively.

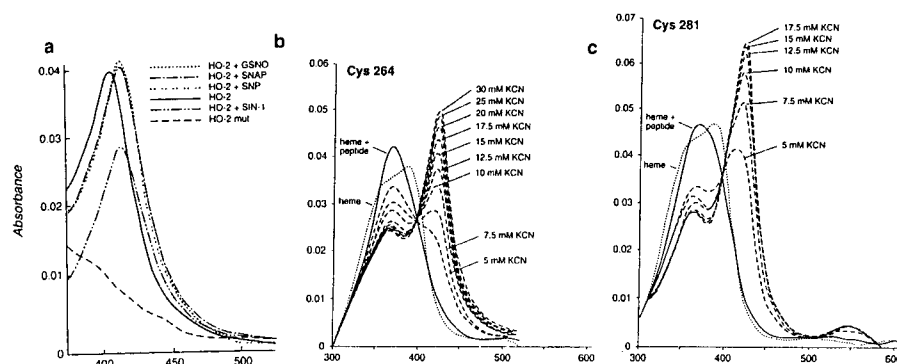


Figure 5. Heme absorption spectrum of HO-2 following treatment with NO donors and comparative affinity of HO-2 heme regulatory motifs and cyanide for heme. Panel a: Purified wild-type HO-2 (2 μ M) was incubated in the absence or presence of the indicated NO donors (100 μ M) at 25°C for 1 h in the dark. The absolute absorption spectrum was recorded using incubation buffer containing the same concentration of NO donor as reference. The spectrum of purified mutated HO-2 protein (HO-2-mut) is also shown. In this protein cysteines 264 and 281 were replaced by alanine. Panels b and c: 10 residue long peptides corresponding to HO-2 sequences that contain Cys264-Pro265 or Cys281-Pro282 were used to analyze the affinity of HO-2 HRMs for heme. The difference absorption spectrum of a 1.2 μ M solution of heme in 0.1 M Tris-HCl (pH 7.5), containing 0.01% Tween-20, was measured over the range of 350 to 360 nm at a scanning rate of 2 nm/Sec. Peptide was added in a stepwise manner to obtain a complete shift of the Soret absorption band. The maximum shift was observed at a heme:peptide 1:4 molar ratio. Incremental additions of KCN solution were then made and the absorption spectrum was determined following each addition until the complete shift of the Soret band to 422 nm was reached, which is that of KCN:heme complex. S-nitroglutathione (GSNO), Sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1).

Effect of PBN pretreatment on tissue levels of ferritin, HO-1 and iron in ischemic/ reperused organ

The next series of experiments examined the effect of pretreatment with the spin trap agent on tissue morphology and expression of genes involved in iron metabolism. As shown in Figure 6, there was a marked difference in immunohistochemical staining for HO-1 (a & b), ferritin (c & d), and transferrin (e & f) in the renal cortex of rats subjected to ischemia/reperfusion in the absence (panels a, c, e) or presence (panels b, d, f) of PBN pretreatment.

As shown, overall, immunostaining for ferritin exhibited the propensity to follow the distribution of HO-1 immunoreactivity. In PBN pretreated rats, increased intensity of HO-1 immunolabeling was noted within the cortex in the proximal and distal tubules. In the absence of PBN pretreatment the cortical region displayed lower intensity of HO-1 immunoreactivity when compared to PBN pretreated rat tissue. Prominent cellular immunostaining for transferrin was noted in the tubules.

Tissue iron staining and iron mediated free radical formation, as reflected by lipid peroxidation, was also examined (Figure 7) by histochemical staining with Schiff's reagent. In PBN pretreated ischemic/reperfused kidney, iron distribution distinctly differed from that observed in the absence of pretreatment and resembled that of sham-operated rat kidneys (data not shown). As noted, staining was confined to the cortical region. As noted, in PBN pretreated rats, staining for iron was largely intratubular (within proximal tubules) and closely followed the distribution of HO-1 and ferritin immunoreactive profiles shown in Figure 6. The intensity of levels of iron staining was visibly lower in vehicle-treated tissue, however, diffuse tissue staining for iron was detected within the renal cortex. In the absence of PBN pretreatment, extensive lipid peroxidation was detected within medullary rays. PBN-pretreated rats did not display lipid peroxidation-mediated Schiff reaction.

Evaluation of morphology of ischemic/reperfused kidney (Figure 8) revealed an overall picture of intact tubules and structure in the presence of PBN pretreatment, whereas in absence of PBN the tubules displayed enlarged lumen size and distorted morphology as indicated by vimentin pattern of tissue staining. In general, the lumen of tubules of ischemic/reperfused kidney in the absence of PBN was visibly enlarged in comparison to that of PBN pretreated rat tissue. Also, in the absence of PBN, increased iron staining was present in distal tubular epithelia of the straight portion of the proximal tubules.

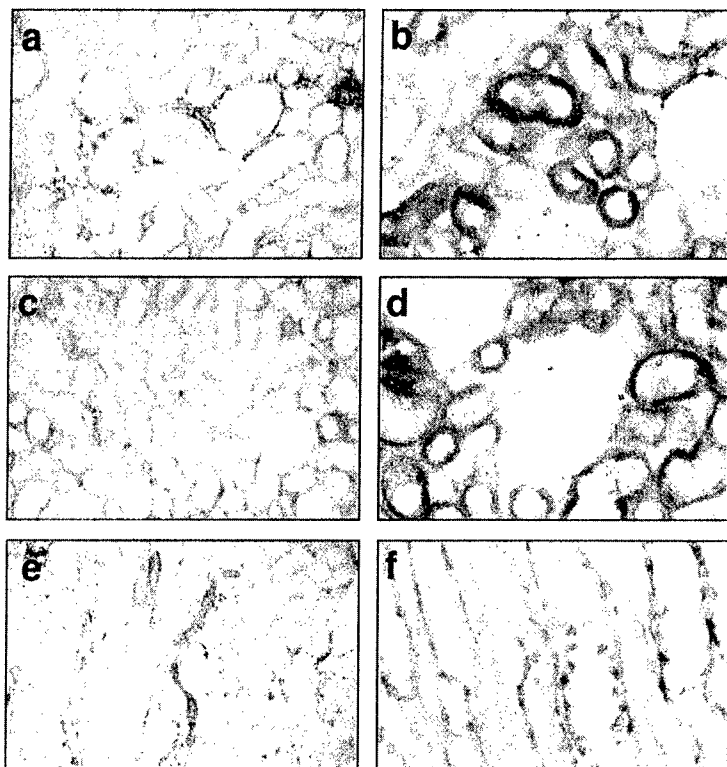


Figure 6. Comparative renal cortical staining for HO-1, ferritin and transferrin of ischemic/reperfused kidney in the presence or absence of PBN pretreatment. Rats were treated with DMSO or PBN dissolved in DMSO (100 mg/kg i.p.) 30 min before bilateral ischemia (30 min) followed by 24h reperfusion. Kidney sections (25 μ m) were obtained and used for histochemical analysis. Detailed description of histochemical experiments is provided in Experimental Procedures. Panels: a, c, e = ischemia/reperfused; panels: b, d, f = PBN pretreated; panels: a & b = HO-1; c & d = ferritin; e & f = transferrin.

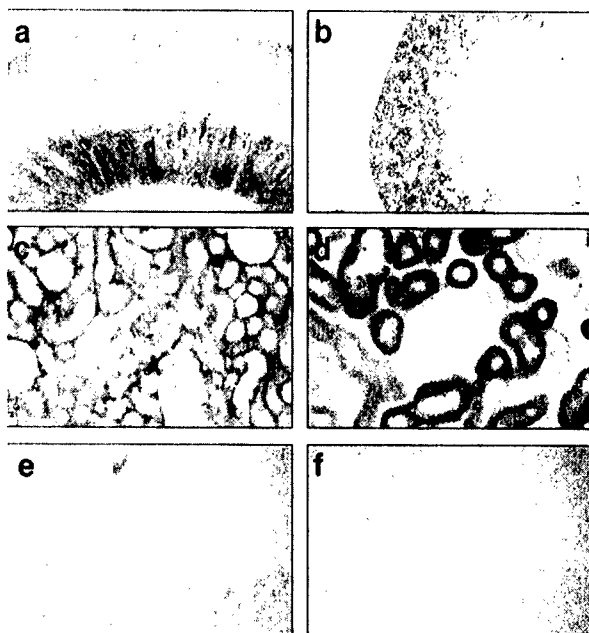


Figure 7. Comparative renal cortical staining for iron and lipid peroxidation of ischemic/reperfused kidney in the presence or absence of PBN pretreatment. Kidney tissue from rats treated as described in Fig 7 were used for iron and lipid peroxidation. Panels a-d = iron staining; panels e and f = Schiff's staining for lipid peroxidation. Panels a, c, e = ischemia/reperfused without PBN pretreatment; panels b, d, f = PBN pretreated rat kidney. Panels a & b = iron, low magnification (x10); c & d = iron, high magnification (x40); e & f = lipid peroxidation, low magnification (x10).

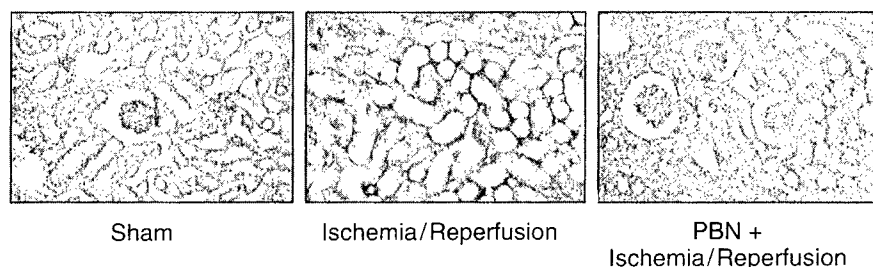


Figure 8. PBN pretreatment protects tissue morphology in ischemia/reperfused rat kidney as indicated by Vimentin staining. Kidney tissue from rats treated as described in Fig 6 were used for Vimentin staining.

Colocalization of increased HO-1 expression with oncogenes

In the next series of the experiments, we examined whether HO-1 increased expression is also involved in tissue defense in case of severe injury inflicted by the compression of the spinal cord. Our previous studies have shown that increased neuronal expression of HO-1 is accompanied by an increase in cGMP levels. Presently 16 h after spinal cord injury (SCI), cGMP immunoreactivity exhibited a pattern of widespread nuclear staining in sampled areas proximal (above) and distal (below) to the site of SCI (Figure 9). Because in cases of SCI neuronal cell loss occurs distal to the site of injury, the expression of those genes that are effectors in cell death was examined. For this analysis, double immunostaining with HO-1 was employed. The heme degradation product, iron, is a potent catalyst for oxygen radicals, histochemical analysis was also carried out for Fe^{3+} . In the first set of experiments, expression of P53 and bcl2 subsequent to SCI was examined (Figure 10) and in the second set of experiments, colocalization of HO-1 with Rip, Bax and Trail was examined (data not shown). These genes were selected because of their responsiveness to cytokines and free radicals. A marked increase in expression of all oncogenes was seen 4-24h after SCI in the neuronal cell body that colocalized with HO-1 in nearly all cells in the spinal cord segments distal to the level of spinal cord compression. Interestingly, there was also a notable increase in iron staining in tissue below the site of injury (data not shown).

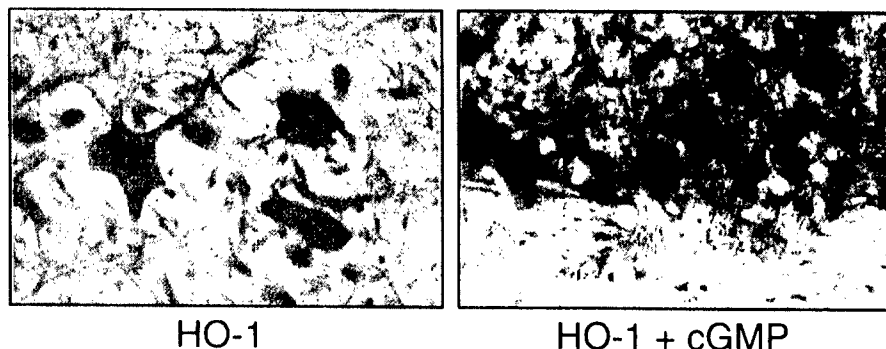


Figure 9. Colocalization of HO-1 with cGMP in the spinal cord neurons. Specimens of the spinal cord below the site of compression injury were double immunostained with anti-HO-1 and anti-cGMP antibodies or with HO-1 antibody alone as described in the Materials and Methods section.

DISCUSSION

Studies using gene “knock in” “knock out” approaches have provided good evidence for both HO-1 and HO-2 being effective components of cellular defense mechanisms (8,10,55,71,76). Presently, based on results obtained with HO-1 and HO-2 primary characteristics and gene regulation, the hypothesis is forwarded that the isozymes although both catalyze the same reaction for oxidation of heme molecule, they have additional functions in cellular defense mechanisms, which reflect those criteria. Specifically, we suggest that the transient HO-1 upregulation is the first line of defense against tissue injury and its sustained increase augments its protective effect. HO-2, on the other hand, is the “house keeping” partner that guards against free radicals at its own expense. The suggestion is supported by findings of *in vitro* experiments with HO-2 protein and NO derivatives (Table 1, Figure 6), and data obtained with PBN pretreated rat kidney subjected to ischemia/reperfusion.

As noted earlier, heme degradation products, i.e. biliverdin and CO, along with bilirubin are considered as positive contributors to cellular defense mechanisms and functions, serving as effective antioxidants and modulators of protein phosphorylation and cell signaling. HO-1 and HO-2 share these dimensions of contribution to cellular defense mechanism. However, because of the multiplicity of cellular events that can be affected directly or indirectly by bile pigments and CO, it is difficult to differentiate the relative contribution of CO-mediated effects and the antioxidant and anticomplement actions of bile pigments to the apparent PBN-mediated enhanced tissue defense against ischemic/reperfusion injury. It is, however, intriguing that suprainduction and the sustained elevation of HO-1 mRNA levels along with

intact HO-2 mRNA levels in PBN pretreated ischemic/reperfused kidney coincides with apparent full protection against tissue damage. Normally, HO-1 mRNA is rather unstable in the cell and its levels return to prestressed levels within a few short hours after induction (18). The mechanism by which PBN protects HO-1 and HO-2 mRNA, likely reflects the activity of the compound to inactivate damaging free radicals. The spin trap scavenge free radicals give rise to relatively stable radicals (14,57); stable radicals with a prolonged half life are more effective in modulating gene expression by the virtue of their prolonged presence in the cell. Reactive oxygen species, including those of nitric oxide and oxygen are effective HO-1 inducers (9,22,23). Free radicals also can cause nucleotide strand breaks.

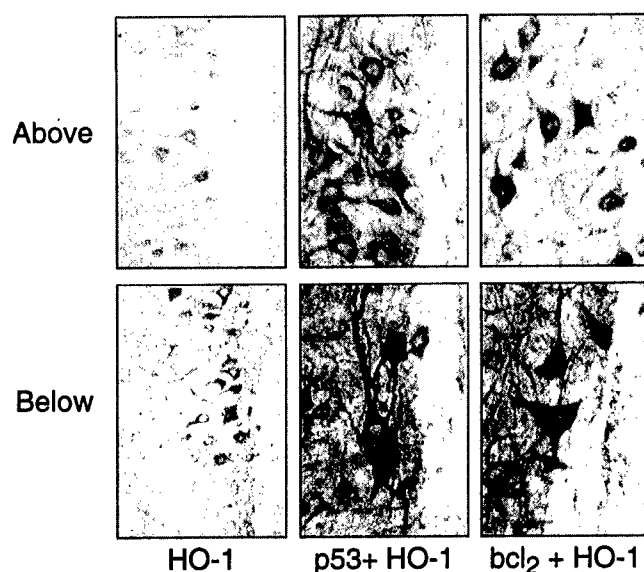


Figure 10. Colocalization of increased expression of HO-1 with oncogenes P53 and bcl_2 . After compression-mediated injury 0.5 cm segments of the mouse spinal cord above and below the site of injury were obtained and used for double immunostaining for HO-1 and apoptotic effector proteins: P53 and bcl_2 . Immunostaining for HO-1 is also shown. Experimental procedures are described in the Materials and Methods section. DAB was used for HO-1 immunostaining; Vector SG chromogen was used for oncogene immunostaining. Note: The original photo was in color. In this black and white reproduction, the light staining is that of HO-1 and the dark staining is that of P53 or bcl_2 .

Therefore, by trapping the powerful oxidizing radical species, the rate of decay in HO-1 mRNA could decrease and breakdown of HO-2 mRNA would be blocked.

Data, showing colocalization of induced HO-1 with an increased pro-apoptotic gene expression, allows for suggestion that the inductions are related and that when tissue is damaged beyond repair, as in the case of

spinal cord compression injury, then the marked increase CO production activates the cGMP-dependent cascades of cell signaling, including the pathways that culminate in cell death by apoptosis. In addition, the MAPKinase pathways which activate apoptotic genes are activated by NO and oxygen radicals (6). Based on the observed increase in redox active iron staining of the spinal cord below the site of compression, together with the fact that Fe^{3+} is a potent catalyst for oxygen derived radicals that can signal the activation of pro-apoptotic genes, it is reasonable to suggest that increased expression of HO-1 is causally linked to the expression of oncogenes, p53, bcl₂, Rip, Bax, and Trail. Indeed, a recent study has shown activation of Caspase 1 by CO and inhibition of cell death by apoptosis when Caspase 1 activity was inhibited (78). We argue that this would constitute a protective mechanism because an important dimension of secondary injury in cases of severe trauma is necrotic cell death, which promotes inflammation. Unlike necrotic cells, cells killed by apoptosis will be cleared in situ by autodestruction, without inducing inflammation and damage to the surrounding tissue.

A major contributing factor to tissue damage after insult is increased in production of NO by the inducible NO synthase and infiltrating macrophage. As documented, it is primarily the activity of NO reaction products that cause tissue damage (13). Therefore, NO derivatives if unchecked would cause inactivation/ destruction of the cellular constituents. It follows, the interaction of HO-2 with, and binding of NO, could protect against NO derived reactive species. The biological significance of NO interaction with HO-2 could be further reasoned in context of the fact that NO being a free radical readily diffuses through most cell barriers and, if it is not consumed, it can interact with oxygen to generate toxic radicals such as peroxynitrite (3,13,88). To control this, it is reasonable to suspect that the cell is equipped with mechanisms, which could include a "sink", by which NO levels are controlled and excess NO is absorbed. Extracellularly, hemoglobin appears to function in this capacity. The cell contains a variety of hemoproteins, which could conceivably serve as a "sink" for NO. However, most hemoproteins are not ubiquitously present in all cell types and/or may not be available to NO. HO-2 would appear to be a good candidate for such a function because: it is present in every cell, and in some, such as the neurons and the germ cells, at impressively high levels (16,17), it is cytoplasmic, and as far as it has been examined, it colocalizes with NO synthase expressing cells (82). As such, then the high level cellular expression of HO-2 could serve to buffer and absorb excess NO generated in the cell. Because with all NO donors there is a decrease in HO-2 activity, this concept could be carried out one step further to suggest that HO-2 is a "suicide sink" for NO. Moreover, we had previously hypothesized (34) on a dynamic link between CO and NO generating systems with NO being an inducer of HO-1 and HO-2 being a "sensor" of heme/heme ligands (i.e O₂, CO, NO) and possibly a

reservoir for heme and heme ligands. The findings of this study are supportive of such an intimate link between the two systems.

In closing, the present findings are supportive of the proposal that in addition to catalyzing CO and bilirubin formation, HO isozymes have disparate functions in defense mechanisms. HO-1 may afford protection against tissue damage by prompt response to the increased demand for heme catalysis, as well as contributing to controlled cell death through intrinsic suicide program, while HO-2, and possibly HO-3, play a major role by sequestering NO derived radicals and suppressing inflammatory response.

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Chapter 18

Hypoxia-inducible factor in brain

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Abstract: HIF-1 is composed of HIF-1 α and HIF-1 β protein subunits. HIF-1 is induced by hypoxia and binds to promoter / enhancer elements and stimulates the transcription of hypoxia-inducible target genes. Because HIF-1 activation might promote cell survival in hypoxic tissues, we studied the effect of stroke on the expression of HIF-1 α , HIF-1 β and several HIF-1 target genes in adult rat brain. After focal cerebral ischemia, mRNAs encoding HIF-1 α , glucose transporter-1 and several glycolytic enzymes including lactate dehydrogenase were up-regulated in the areas around the infarction. HIF and its target genes were induced by 7.5 hours after the onset of ischemia and increased further at 19 and 24 hours. Since hypoxia induces HIF in other tissues, systemic hypoxia (6% O₂ for 4.5h) was also shown to increase HIF-1 α protein expression in the adult rat brain. It is proposed that decreased blood flow to the penumbra decreases the supply of oxygen and that this induces HIF-1 and its target genes. Because HIF-1 activation may promote cell survival in hypoxic tissues, we studied the effect of hypoxic preconditioning on HIF-1 expression in neonatal rat brain. Hypoxic preconditioning (8% O₂/3hrs), a treatment known to protect the newborn rat brain against hypoxic-ischemic injury, markedly increased HIF-1 α and HIF-1 β expression. We also studied the effect of two other known HIF-1 inducers, cobalt chloride (CoCl₂) and desferrioxamine (DFX), on HIF-1 expression and neuroprotection in newborn brain. HIF-1 α and HIF-1 β protein levels were markedly increased after i.p. injection of CoCl₂ and DFX. Preconditioning with CoCl₂ or DFX 24 hours before the stroke decreased infarction by 75% and 56% respectively, compared with vehicle-injected, littermate controls. Thus, HIF-1 activation could contribute to protective brain preconditioning.

Key Words: hypoxia, hypoxia-inducible factor, ARNT, pre-conditioning, ischemic tolerance, lactate dehydrogenase, stroke, cerebral ischemia

INTRODUCTION

During ischemia to any organ, decreased blood flow limits the supply of glucose and oxygen. The lack of oxygen stimulates the expression of many genes. Many of these hypoxia-inducible genes appear to share a common mode of regulation that involves activation of a transcription factor called hypoxia-inducible factor 1 (HIF-1,14,55,56). HIF-1 is a nuclear protein heterodimer (65) that binds to a bipartite consensus sequence in hypoxia-responsive enhancers / promoters of many target genes including erythropoietin, vascular endothelial growth factor (VEGF), inducible NOS, heme oxygenase-1, glucose transporter-1 and several if not all of the glycolytic enzymes (11,57,72).

HIF-1 is a heterodimer composed of two basic helix-loop-helix PAS domain (bHLH-PAS) proteins initially called HIF-1 α and HIF-1 β (or ARNT, aryl hydrocarbon nuclear receptor translocator) (70). It is now known that there is a HIF-2 α , alternative splice variants of HIF-1 α , and two ARNTs, ARNT1 and ARNT2 (22,48,42). Whereas HIF-1 α is specific to the HIF-1 heterodimer, HIF-1 β has several cellular functions other than being a component of HIF-1. HIF-1 β also dimerizes with other bHLH-PAS proteins such as the aryl hydrocarbon receptor (26), and the HIF-1 α homologue, HIF-2 α (13). HIF-1 α exhibits very high affinity for HIF-1 β resulting in competition with the aryl hydrocarbon receptor for the recruitment of HIF-1 β (23). Upon dimerization with HIF-1 β , HIF-1 α acquires a conformation that renders it more resistant to proteolytic digestion (38).

HIF-1 α mRNA and protein and HIF-1 DNA-binding activity are induced by hypoxia (74). HIF-1 is induced by decreased molecular oxygen (53), and appears to be induced by free radicals produced in mitochondria complex I since MPTP suppresses hypoxia induction of HIF-1 in brain (1). The oxygen sensing mechanism for HIF-1 appears to involve a oxygen-binding heme protein possibly in the mitochondrial (24,53). In contrast, HIF-1 β mRNA expression is marginally affected by decreased oxygen. HIF-1 α is a short-lived PEST protein rapidly degraded under normoxic conditions by the ubiquitin-proteasome system (52,38), with the Von Hippel Lindau protein playing a key role in targeting HIF to the proteasome (46,60). During hypoxia, degradation is blocked resulting in HIF-1 α protein accumulation. Because induction of HIF-1 DNA-binding activity can be inhibited by pretreatment of hypoxic cells with actinomycin-D or cyclohexamide, RNA transcription and protein synthesis may be necessary for HIF-1 activation (54). Phosphorylation also appears necessary to activate HIF-1 activity (67,2). Because activation of HIF-1 may promote survival of vulnerable hypoxic tissue by increasing glucose transport and glycolysis, we studied the effect of permanent focal cerebral ischemia on the expression of HIF-1 and

several of its target genes in adult rat brain in the first part of the studies reported here and described previously (4).

In the second part of the studies described below, we examined HIF as potential mechanism for hypoxia-induced pre-conditioning. Preconditioning is a process by which a tissue is rendered more tolerant to a subsequent lethal insult such as ischemia. Tolerance can be attained by subjecting tissues to a sublethal stress that results in intracellular adaptation and enhanced endogenous defense mechanisms. In the brain, neuronal tolerance against ischemic injury has been reported after preconditioning with a brief sublethal episode of ischemia, spreading depression or exposure to some chemicals (7,71). In addition, hypoxia preconditioning has been shown to produce tolerance against hypoxic-ischemic brain injury in newborn rats (20,21). Induced-tolerance has also been observed in rat hippocampal slices preconditioned by anoxia (49). Interestingly, despite several studies on hypoxia-induced tolerance in rodent brain, the mechanisms underlying this neuroprotection remain poorly understood. Because it generally takes several hours to days after the preconditioning episode before observing a state of tolerance, it suggests that the preconditioning stimulus is likely to involve adaptive changes in gene expression. Accordingly, using a reproducible model of hypoxia-induced tolerance in the immature rat, the second part of the studies described below were performed in order to start elucidating the molecular mechanisms underlying this hypoxic preconditioning.

We did this by giving chemicals that are known to induce HIF. The divalent metal cobalt chloride (CoCl_2) and the iron chelating agent desferrioxamine (DFX) also stimulate HIF-1 expression, HIF-1 DNA-binding activity, and the transactivation of several HIF-1 target genes under normoxic conditions (29,30). Though the mechanism of action of these compounds is still unclear, they appear to interact with the heme/iron protein oxygen sensor (53). We first determined whether cobalt chloride and DFX induced HIF in brain, and then whether the compounds protected the brain against stroke.

RESULTS

In situ hybridization autoradiography showed that HIF-1 α and HIF-1 β mRNAs were expressed throughout the normal brain. Following strokes produced by middle cerebral artery occlusions using the suture method, HIF-1 α and HIF-1 β mRNA were expressed throughout the normal hemisphere in a distribution identical to that seen in normal, untouched animals or normal, sham-operated animals. Northern blots confirmed that the amount of HIF-1 α

and HIF-1 β mRNA in the normal hemisphere contralateral to the MCA occlusions was comparable to that found in brains from control animals.

HIF-1 α and HIF-1 β mRNA were detected throughout all of cortex, including cingulate, entorhinal, frontal, occipital, parietal and temporal cortex. Constitutive expression of both mRNAs was also observed in the caudate putamen, thalamus, hypothalamus and all forebrain structure. Expression was high in medial habenula and particularly low in the white matter of the corpus callosum and internal capsule. HIF-1 α and HIF-1 β mRNAs were constitutively expressed at high levels in hippocampus, including the CA1 and CA3 pyramidal cell layer as well as dentate granule cell layer. Preliminary cellular studies suggested that HIF-1 mRNAs are expressed in most if not all neurons in the brain.

Western immunoblots also confirmed that there is constitutive expression of both HIF-1 α and HIF-1 β protein in the cingulate/retrosplenial cortex of control untreated animals. These observations are consistent with previous reports of constitutive expression of HIF-1 α and HIF-1 β mRNAs in adult rat and human brains (74) and in embryonic mouse brain (33).

Because HIF-1 is induced by hypoxia, the effects of stroke on HIF-1 expression in rat brain were examined. Permanent middle cerebral artery (MCA) occlusions produced ipsilateral damage throughout the entire MCA territory including parietal and occipital cortex, caudate/putamen, thalamus and hypothalamus. The hippocampus was occasionally damaged as previously described (59). In situ hybridization showed increased HIF-1 α mRNA expression outside the ischemic infarct, in the MCA and anterior cerebral artery territories including the cingulate/retrosplenial cortex, adjacent motor cortex, the entorhinal cortex and part of the medial thalamus. HIF-1 α mRNA induction in the ischemic penumbra was observed as early as 4-7h after permanent MCA occlusion and increased significantly up to 20h (Figure 1, left panel). HIF-1 β mRNA showed a slight decrease at 7.5h following MCA occlusions, but otherwise unchanged over the 20h following MCA occlusions (Figure 1, right panel). Northern blot analysis of mRNA extracted from cingulate/retrosplenial cortex showed a 15-17 fold increase in HIF-1 α mRNA level after 20h of MCA occlusion compared to contralateral non-ischemic or untreated control cortex. In the same extracts, HIF-1 β mRNA was unaffected by ischemia compared with controls. Because induction of HIF-1 requires ongoing protein synthesis and protein stabilization, HIF-1 α and HIF-1 β protein expression were evaluated by Western immunoblotting in the cingulate/retrosplenial cortex of animals subjected to 20h of focal ischemia. MCA occlusion produced a 1.8 to 2.5-fold increase in HIF-1 α and HIF-1 β proteins compared with contralateral or untreated cortex. Since ischemia induced HIF-1, the next experiments determined whether hypoxia alone would induce HIF in brain as it does in other tissues. Rats subjected to systemic hypoxia (6% O₂ for 4.5h)

demonstrated a 2.3 to 3.7-fold increase in the expression of both HIF-1 α and HIF-1 β proteins in the cingulate/retrosplenial cortex. The increase of HIF-1 β protein with both ischemia and hypoxia is notable since ischemia did not appear to induce HIF-1 β mRNA, suggesting that both ischemia and hypoxia may stabilize the HIF-1 α and HIF-1 β protein dimers as discussed below.

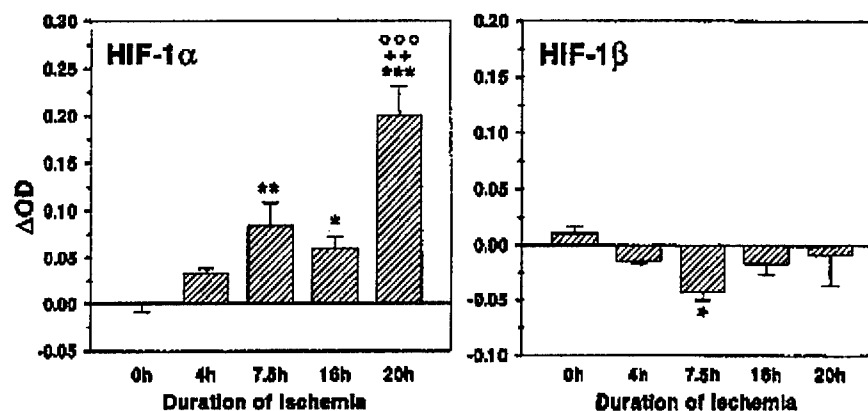


Figure 1. Hypoxia inducible factor-1 α (HIF-1 α) and HIF-1 β mRNA expression in cingulate cortex adjacent to a middle cerebral artery (MCA) occlusion compared to control cingulate cortex in the opposite hemisphere. Optical densities were measured on the ischemic side and control side and the differences of the optical density shown on the y-axis. Times after the MCA occlusions are shown on the x-axis. Note that HIF-1 α mRNA increased at 7.5 hours, 16 hours, and 20 hours following MCA occlusions.

Reduction in oxygen tension results in increased HIF-1 mRNA and protein levels in cultured cells and in isolated perfused and ventilated lung preparations. Because tissue hypoxia may occur when cerebral blood flow is reduced, regional cerebral blood flow was measured in rats subjected to 1 and 24 hour permanent MCA occlusions. As early as 1h after the onset of MCA occlusion, blood flow in the ipsilateral parietal cortex was markedly reduced by 59.3-81.7% of the contralateral value. Similar blood flow reductions were observed in ipsilateral caudate/putamen, lateral thalamus and hypothalamus. In the cingulate/retrosplenial cortex ipsilateral to the MCA occlusion, blood flow was reduced by 9.4-20.3% (mean value: 15.3%) of the contralateral value. At 24 hours following MCA occlusions, blood flow in the infarcted parietal cortex was markedly reduced by 71.4-84.6% (mean value: 78.9%; $p < 0.001$ compared with the 1h group) of the contralateral value. Similar reduction of cerebral blood flow was observed in other infarcted core regions such as the caudate/putamen, hippocampus, and lateral thalamus and hypothalamus. In contrast, blood flow was moderately reduced in the cingulate/retrosplenial cortex by 15.3-35.7% (mean value:

24.2%; $p < 0.001$ compared with the 1h group) of the contralateral value. This region of cortex, which appears to represent the penumbra, consistently showed increased HIF-1 α and HIF-1 β expression. Blood flows in the cingulate / retrosplenial cortex and all other regions of the brain in sham-operated and control rats were identical on both sides of the brain.

The promoter/enhancer region of a number of genes, including the glucose transporter-1 (GLUT-1) and the glycolytic enzymes aldolase-A (ALD-A), enolase-1, lactate dehydrogenase-A (LDH-A), phosphofructokinase-L (PFK-L), phosphoglycerate kinase-1 and pyruvate kinase-M (PK-M) (16), contain at least one HIF-1 binding site. Because the expression of these HIF-1 target genes may be regulated by increased expression of HIF-1 subunits and consequent increased HIF-1 DNA-binding activity, we investigated the effect of permanent focal ischemia on the expression of GLUT-1 and the glycolytic enzymes PFK-L, ALD-A, PK-M and LDH-A. These enzymes were selected because they represent different levels of the glycolytic pathway and because of many reports that they are induced by hypoxia in vitro. GLUT-1 is a facilitated glucose transporter protein that mediates glucose transport mainly across the blood-brain barrier but also into some neurons and glia (64). LDH-A (or LDH-5) is found in both neurons and astrocytes and preferentially converts pyruvate to lactate (5).

In situ hybridization autoradiography showed low constitutive expression of GLUT-1 mRNA in the non-ischemic hemisphere. There was more prominent expression in periventricular locations, the choroid plexus and several blood vessels. PFK-L mRNA was constitutively expressed in cortex, caudate/putamen, thalamus and hypothalamus. The most prominent expression was observed in the pyramidal cell layer of CA1-CA3 and dentate regions of the hippocampus, piriform cortex, medial habenula, paraventricular thalamic nucleus, arcuate periventricular hypothalamic nucleus and the lining of the ventricles. The expression of ALD-A and PK-L mRNA was similar, with the highest signal being detected in the pyramidal cell layer of CA1-CA3 and dentate of the hippocampus, piriform cortex, medial habenula and medial thalamic nuclei. LDH-A mRNA was also expressed throughout brain, with the highest expression being observed in cortex, the pyramidal cell layer of CA1-CA3 and dentate of the hippocampus, medial habenula, the paraventricular thalamic nucleus, and medial thalamic and hypothalamic nuclei. There was no difference in the distribution and level of expression of all HIF-1 target genes between the untreated brain and the cerebral hemisphere contralateral to the MCA occlusion.

Following MCA occlusion the in situ hybridization autoradiographs showed that the GLUT-1, PFK-L, ALD-A, PK-M and LDH-A mRNAs increased in the same regions where HIF-1 α expression was increased after

MCA occlusions. The levels of the HIF-1 target gene mRNAs increased in the MCA and anterior cerebral artery territories including the cingulate/retrosplenial cortex, motor cortex and parts of medial thalamus. GLUT-1 and LDH-A mRNA increased by 7.5 hours following MCA occlusions, with smaller but not significant induction of PFK-L and PK-M mRNAs. By 19 to 24 hours following permanent MCA occlusions all of the HIF-1 target gene mRNAs had markedly increased including LDH-A (Figure 2).

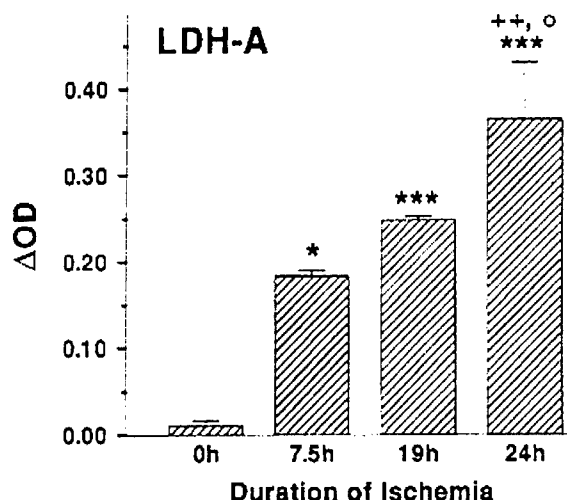


Figure 2. Lactate dehydrogenase mRNA expression in cingulate cortex adjacent to a MCA occlusion compared to control cingulate cortex in the opposite hemisphere. Note that LDH-A mRNA expression increased at 7.5 hours, 19 hours and 24 hours after the MCA occlusions.

The next series of experiments were performed in the 7 day old rat, because Gidday and colleagues had previously shown that a 3 hour exposure to 8% oxygen protected the brain of these animals against stroke (hypoxia-ischemia) 24 hours later (20). Northern blots showed that HIF-1 α and HIF-1 β mRNA were constitutively expressed at low but detectable level consistent with previous studies in normal adult rat and human brains. Similarly, low levels of HIF-1 α and HIF-1 β proteins were detected in control brain tissue by Western blot analysis. Immunohistochemistry experiments also confirmed that there is constitutive HIF-1 α expression throughout the newborn rat brain with more intense staining in the molecular layer of CA1-CA3 subfields of the hippocampus and in layers 1 to 3 of the cerebral cortex. Moderate HIF-1 α immunostaining was also noted in the striatum, the ventral thalamic nucleus, the periventricular and ventromedial hypothalamic nuclei.

Newborn rats exposed to hypoxia 1 day before ischemia are protected against ischemic brain injury. Because HIF-1 is induced by hypoxia, we

investigated the role of HIF-1 in hypoxia-induced ischemic tolerance in brains from rat pups preconditioned by exposure to 8% O₂ for 3 hours. Because HIF-1 is degraded rapidly upon reoxygenation, brains were analyzed immediately after the end of hypoxia exposure, before reoxygenation. Northern blot analysis of brain tissue after preconditioning showed a 1.7 to 2.5-fold increase in HIF-1 α mRNA level whereas HIF-1 β mRNA level was unchanged compared with untreated control. Because induction of HIF-1 activity requires ongoing protein synthesis and protein stabilization, HIF-1 α and HIF-1 β protein expression was evaluated by Western blot analysis. The hypoxia preconditioning produced a 25.8 to 62.9-fold increase HIF-1 α protein levels and a 2.5-5.9-fold increase in HIF-1 β protein levels. In animals subjected to hypoxia-ischemia, similar changes in both HIF-1 proteins were observed in the contralateral hemisphere which was exposed to hypoxia only.

We then investigated the effect of combined unilateral common carotid occlusion and systemic hypoxia in newborn rat brain. This model of perinatal hypoxia-ischemia in rat pups produces severe oxygen depletion and subsequent brain injury in the hemisphere ipsilateral to the carotid occlusion. Northern blot analysis of brain extracts obtained from the ipsilateral (hypoxic-ischemic) hemisphere showed 1.4 to 2.8-fold increase in HIF-1 α mRNA whereas HIF-1 β mRNA level remained unchanged compared with untreated controls. Western blot analysis also showed a modest 1 to 11.4-fold increase in HIF-1 α protein level and unchanged or slightly decreased (0.2 to 0.34-fold) HIF-1 β protein level after hypoxia-ischemia compared with untreated control. This induction of HIF-1 α protein expression observed after hypoxia-ischemia was always several fold smaller than that produced by hypoxia alone in the contralateral hemisphere or by hypoxia preconditioning.

To further investigate the role of HIF-1 in hypoxia preconditioning, we studied the effect of known HIF-1 inducers other than hypoxia. In vitro studies have shown that HIF-1 is strongly induced by DFX or CoCl₂. Therefore, we examined HIF-1 expression in brains from rat pups administered either DFX or CoCl₂. There was no overt adverse physiological or behavioral change in newborn rats administered either DFX or CoCl₂ compared with vehicle injected animals. In particular, animals receiving DFX or CoCl₂ demonstrated intact grasping and righting reflexes, normal ambulation, respiration, suckling and tail pinch response. Brains from these animals showed no abnormalities on Nissl staining. Western blot analysis showed that DFX treatment produced a small 1.3 to 2.4-fold increase in HIF-1 α protein level whereas HIF-1 β levels were elevated 0.7 to 2.1-fold in 7 out of 13 animals treated with DFX, irrespective of the exposure time. In contrast, CoCl₂ administration resulted in increased HIF-1 α (0.5 to 4.3-fold) and HIF-1 β (9.4 to 10.0-fold) proteins 1 hour after injection compared with

time matched vehicle-injected controls. At 3 hours post-injection, peak levels of HIF-1 α (4.3 to 38.7-fold) and HIF-1 β (9.7 to 17.4-fold) proteins were observed compared with the corresponding control. Six hours after CoCl₂ injection, HIF-1 α and HIF-1 β protein levels showed a 38% decline compared with the levels found at the 3 hours time point.

To evaluate the relationship between the level of HIF-1 expression and tolerance against ischemic brain damage, we investigated whether DFX and CoCl₂ pretreatment (that induce HIF-1 expression) reduced hypoxic-ischemic brain injury in newborn rats. Significant infarction, manifested as 32.3 ± 4.0 % (vehicle group, no DFX; Figure 3, left panel) and 43.0 ± 3.7 % (vehicle group, no CoCl₂; Fig. 3, right panel) reductions in hemispheric weight ipsilateral to the carotid ligation, was noted in vehicle-treated animals one week after hypoxia-ischemia. These observations are consistent with previous reports (20,19,21). Preconditioning with DFX or CoCl₂ resulted in a 14.1 ± 3.0 % and 10.6 ± 3.4 % loss in ipsilateral hemispheric weight which represent a 56% (DFX) and 75% (CoCl₂) protection compared with vehicle-injected controls (Figure 3).

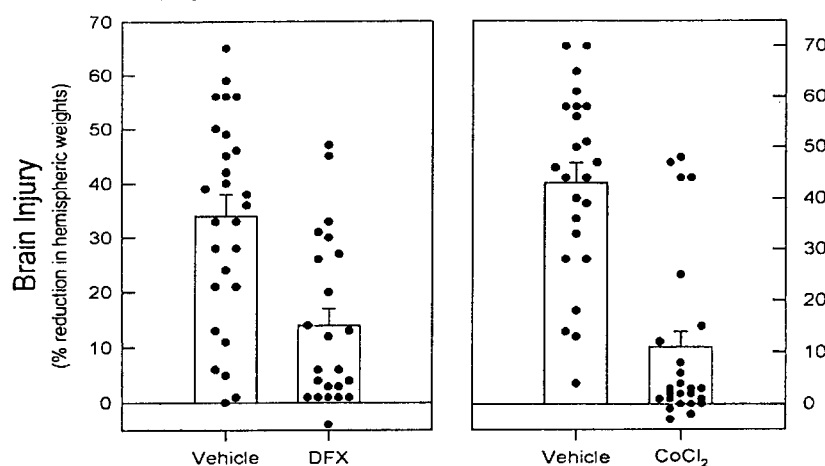


Figure 3. Hypoxia-ischemia in the 7 day-old rat produced 35 to 40% reductions in the hemispheric weight in vehicle injected controls. Treatment with desferrioxamine or with cobalt chloride markedly decreased the ischemic injury, with a majority of the cobalt chloride treated animals showing no evidence of injury.

DISCUSSION

The results of the adult rat study demonstrate that focal cerebral ischemia induces HIF-1 α mRNA and increases the levels of both HIF-1 α and HIF-1 β proteins. The up-regulation of HIF-1 is restricted to the tissue surrounding

the ischemic infarct and extends to adjacent regions supplied by the anterior and posterior cerebral arteries. We postulate that tissue hypoxia, produced by a moderate decrease in cerebral blood flow, induces HIF-1 in the penumbra.

Hypoxia induces HIF-1 expression and DNA-binding activity in mammalian cell lines (34) and in different mouse, rat and ferret organs (74,76). The HIF-1 response in Hela cells increases exponentially as oxygen concentrations fall below 6% (76), with HIF-1 α protein levels increasing 2-5 times more than HIF-1 β protein levels (37). HIF-1 α mRNA levels increase dramatically in the lung with decreased inspired O₂ concentration whereas HIF-1 β mRNA is not induced (76). HIF-1 α but not HIF-1 β mRNA is similarly upregulated in ischemic mouse retina (47) and in brain following global ischemia (36). This is consistent with data that suggests that HIF-1 α protein levels determine HIF-1 DNA-binding activity and transcription during hypoxia (Jiang et al., 1996; Semenza et al., 1996). In the present study, HIF-1 α but not HIF-1 β mRNA increased in the peri-infarct regions. This induction could be dependent on the extent and/or the duration of reduced cerebral blood flow and decreased delivery of oxygen in the penumbra after permanent focal ischemia.

Though HIF-1 α mRNA was markedly induced following stroke, HIF-1 α protein level was only moderately increased. In addition, the predominant protein species accumulating after ischemia migrated to a higher position in the gel suggesting that post-translational modification of HIF-1 α occurred (66). HIF-1 α splice variants have been described in humans as well that have DNA binding activities (22). Relatively modest induction of HIF-1 α protein despite high levels of HIF-1 α mRNA may indicate inadequate translation of the HIF-1 α protein. Alternatively, it could relate to changes in the ubiquitin-proteasome HIF-1 α degradation system that might occur with altered redox conditions in the penumbra (52,29,38,60,46). The absence of hypoxia-induced modulation of HIF-1 β mRNA was expected because HIF-1 β (ARNT) contributes to other multimeric transcription factors, besides HIF-1, that are involved in gene regulation unrelated to hypoxia (12,17,27,62,72). In addition, there are two ARNTs, 1 and 2, with ARNT2 being expressed specifically in neurons, and possibly not being related to a hypoxia response in those cells (9). The moderate increases of HIF-1 β protein levels following stroke, without increases in the mRNA, are consistent with decreased degradation and stabilization of HIF-1 β protein that has been observed with previous *in vivo* and *in vitro* hypoxia studies (38,76).

The mean pO₂ value in normal mammalian cerebral cortex (73) and other organs (35) is estimated to be 2% to 5%. This moderate tissue hypoxia might explain the constitutive expression of mRNA and protein encoding the HIF-1 subunits throughout normal rat brain, and in other human and rodent tissues (33). Thus, even modest reductions in oxygen concentrations in brain

might induce HIF-1 because it would occur in the steep part of the exponential dose response curve (35). Indeed, increased HIF-1 expression occurs in several organs including brain of mice and rats subjected to systemic hypoxia (74). Up-regulation of HIF-1 in brain by systemic hypoxia in this study is consistent with the hypothesis that hypoxia is a primary stimulus for HIF-1 induction in all tissues including brain (74). However, recent studies suggest that a number of compounds that appear to produce or regulate the production of reactive oxygen species modulate HIF expression, including MPTP, angiotension, PDGF, IL1, and others (1,6,50,41,61).

Tissue hypoxia may result from a reduction in cerebral blood flow. The penumbra has been described as an area in which regional cerebral blood flow is approximately 20-50% of normal, which is the threshold level below which synaptic transmission fails (28). Consistent with previous studies, blood flow in the penumbra was reduced 9.4-20.3% of contralateral values at one hour after permanent MCA occlusion, and was maintained at similar levels (15.3-35.7%) for up to 24 hours. Despite the early decrease in blood flow in the penumbra, significant induction of HIF-1 α mRNA expression was observed only after 7.5h of permanent focal ischemia. This is consistent with other findings that show that HIF-1 increases after 8h of continuous hypoxia (76).

HIF-1 mRNAs and proteins were not induced in regions of the MCA territory that go on to infarct presumably because blood flow reduction in the core regions was rapidly reduced to a level that is not compatible with transcription or translation (39). In contrast, because the reduction of blood flow in the penumbra area is moderate, increased time of exposure to hypoxia may be required in order to observe significant tissue hypoxia and HIF-1 activation. Increased oxygen extraction in the hypoxic tissue may also contribute to the delayed HIF-1 response in the penumbra (25).

Deletion and mutational analyses demonstrate the presence of at least one functionally active HIF-1 binding site in the promoter/enhancer region of HIF-1 target genes encoding the glucose transporter-1 (10) and the glycolytic enzymes (15,16,57). Disruption of the HIF-1 α (31,51) or HIF-1 β (43) genes in mouse embryonic stem cells reduces the *in vivo* response of HIF target genes to hypoxia, and impairs physiological responses to hypoxia (77). The hypoxic regulation of GLUT-1 and glycolytic enzymes is altered in mutant cells where HIF-1 α or HIF-1 β are reduced or absent (75). In the present study, HIF-1 induction of GLUT-1, PFK-L, ALD-A, PK-M and LDH-A is supported by the finding that the genes are induced in the same areas, and the finding that HIF-1 is induced either prior to or concurrently with the target genes. This is similar to other studies where HIF-1 is first induced and this is later followed by peak induction of GLUT-1 mRNA at 12 hours following MCA occlusions (63) and HIF-1 target gene induction in different cell lines at 16 hours following continuous exposure to low O₂

(32). HIF-1 induction of GLUT-1 and the glycolytic enzymes could modulate the delayed changes in cerebral glucose utilization that occur following stroke (58).

The survival of cells following oxygen depletion may depend in part on changes in the expression of HIF-1 regulated genes. The oxygen and energy depletion that occurs so rapidly in the core of an ischemic infarct precludes a HIF response. However, the moderate and prolonged decreased blood flow and decreased oxygen supply that occurs in the penumbra appears to lead to induction of HIF-1. This may promote the survival of this functionally disturbed but still viable tissue by increasing glucose transport and glycolysis.

The studies in the neonatal rat brain addressed the question of whether HIF might be involved in the hypoxia-induced ischemic tolerance as originally described by Gidday (20). Cells respond to hypoxia by modulating the expression of adaptive or pathological genes depending on the severity and duration of the stimulus. Newborn rats subjected to hypoxia preconditioning (8% O₂/3 hrs) sustain markedly reduced brain damage after cerebral hypoxia-ischemia compared with animals not exposed to prior hypoxia (20,19,21). Such preconditioning produces moderate tissue hypoxia which has relatively no long term effects on regional cerebral blood flow, water content, calcium uptake, protein synthesis and cellular integrity(4). The present study provides evidence that HIF-1 induction may be an important component of the adaptive response elicited by hypoxia preconditioning in neonatal rat brain. Levels of HIF-1 α mRNA and of both HIF-1 α and HIF-1 β proteins were markedly increased throughout the newborn rat brain immediately after hypoxia preconditioning. In addition, preconditioning with either CoCl₂ or DFX induced HIF-1 expression and resulted in neuroprotection.

In newborn rat brain, the expression of several genes such as the immediate early genes *fos* and *jun* and the stress proteins HSP72 and HSP32 is unaffected by hypoxia treatment whereas combined hypoxia-ischemia results in a marked upregulation of these genes (45). In the present study, HIF-1 does not follow the classical definition of stress gene as it is induced by hypoxia preconditioning and repressed by hypoxia-ischemia despite a small elevation of HIF-1 mRNA. Hypoxia-ischemia in newborn rat brain produces severe oxygen depletion and decreased energy metabolites which lead to decreased overall protein synthesis in the ischemic brain parenchyma. Since HIF-1 activation requires *de novo* protein synthesis, an impairment of protein synthesis during hypoxia-ischemia may account for the lack of induction of HIF-1 protein expression in the ischemic newborn brain. Interestingly, the marked expression of HIF-1 α protein in the microvasculature of the ischemic core region support the role of HIF-1 in angiogenesis through upregulation of VEGF (8,18,40,44).

To investigate the protective role of HIF-1 in hypoxia-induced tolerance in newborn rat brain, we tested whether DFX and CoCl_2 , which are two known inducers of HIF-1 expression and activity *in vitro* (68), also induced HIF-1 in rat brain. We also wanted to correlate the induction of HIF-1 with its potential neuroprotective effect. As predicted by the *in vitro* data, administration of either DFX or CoCl_2 increased the level of HIF-1 protein subunits in newborn rat brain. Interestingly, CoCl_2 was a much better inducer of HIF-1 expression than DFX. Whereas CoCl_2 treatment increased both HIF-1 α and HIF-1 β protein levels, DFX treatment resulted in HIF-1 β protein levels several fold greater than the modest increase in HIF-1 α protein level. Because HIF-1 α is the limiting factor which determines the level of HIF-1 DNA binding and transcriptional activity within cells, the small induction of HIF-1 α protein expression by DFX despite increased expression of HIF-1 β may account for the more modest neuroprotection afforded by DFX preconditioning compared with CoCl_2 or hypoxia treatments. Although the levels of DFX or CoCl_2 in neonate brain were not measured in the present study, previous observations suggest that the differences in the ability of DFX and CoCl_2 to stimulate HIF-1 expression may be the result of different blood brain barrier permeability.

In the present study, preconditioning newborn rats with hypoxia or hypoxia-mimetics (DFX and CoCl_2) produced a long lasting adaptive and protective response that is likely to involve increased HIF-1 target genes as a result of HIF-1 activation. Indeed, increased glucose transporter-1 mRNA has been reported in cerebral microvessels after hypoxia and hypoxia-ischemia in newborn rat brain (64) consistent with our findings of increased HIF-1 α in blood vessels following the same treatments. *In vitro*, DFX or CoCl_2 induces the expression of several HIF-1 target genes like erythropoietin, glucose transporter-1, VEGF and the glycolytic enzymes (3,41,69).

In conclusion, the studies in the neonatal rat brain showed that the level of HIF-1 α expression correlated with the degree of brain protection afforded after each preconditioning treatment (hypoxia> CoCl_2 >DFX). Thus increased expression of HIF-1 target genes as a result of HIF-1 activation could induce several defense mechanisms such as increased glucose transport, glycolysis and vascular growth, all of which may contribute to protective brain preconditioning and neuronal survival.

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Chapter 19

Proton-gated cation channels – neuronal acid sensors in the central and peripheral nervous system

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Abstract: Metabolic hyperactivity or limited oxygen supply can cause a decrease of tissue pH. Severe tissue acidosis that accompanies ischemia and most forms of inflammation is painful and sensory neurons respond to acidic tissue pH with increased firing. H⁺-gated cation channels in sensory nerve endings are thought to be responsible for the activation of nociceptive afferents by acid. The members of one family of recently identified H⁺-gated cation channels (ASICs, Acid Sensing Ion Channels) are candidates for the acid sensor in sensory nerve endings. Certain ASIC subunits are also or exclusively expressed in neurons of the central nervous system (CNS) where the role of those cation channels is as for yet unknown. Neuronal activity is accompanied by pH fluctuations and the widespread expression of ASIC channels throughout the CNS suggests that activation of those ion channels by local acidic transients might play a role in neurotransmission or neuromodulation.

Key words: ASIC channels, nociception, sensory neurons, neurotransmission

INTRODUCTION

The pH of most tissues is tightly regulated and remains within a narrow range close to neutral. However limited oxygen supply and metabolic hyperactivity can cause a drop of tissue pH. In ischemic or inflamed tissues even extreme pH values (<pH 6) can be reached (reviewed in (26)). Acidosis signals an abnormal potentially threatening condition and sensing and responding to a tissue acidosis is important for an organism. It is thus not surprising that a subset of sensory neurons disposes of mechanisms to detect

acidic tissue pH (1, 29). Those acid sensing neurons depolarize when the extra cellular pH decreases. However, also many central neurons respond to acidic pH with increased activity (1, 32). The physiological role of the acid sensitivity of central neurons is as yet unknown. Na^+ -permeable cation currents that are activated by extra cellular protons were described in both central and sensory neurons (1, 30, 32). Those H^+ -gated cation currents are thought to be responsible for the firing of neurons exposed to acidic pH. The Acid-Sensing Cation Channels (ASIC) we and others cloned during the past years are candidates for the acid sensor in neurons (reviewed in (36)). H^+ -gated cation channels form a family of ion channels (Figure 1) with distinct tissue distribution and properties. At present, the products of three ASIC genes (ASIC1, ASIC2, ASIC3) were shown to form H^+ -gated cation channels. Alternate splicing of ASIC1 (ASIC1a, ASIC1b) and ASIC2 (ASIC2a, ASIC2b) and heteromultimeric assembly of ASIC subunits further increases the diversity of ASIC channels. The splice variants differ in their amino terminus (about 30 - 40% of the protein).

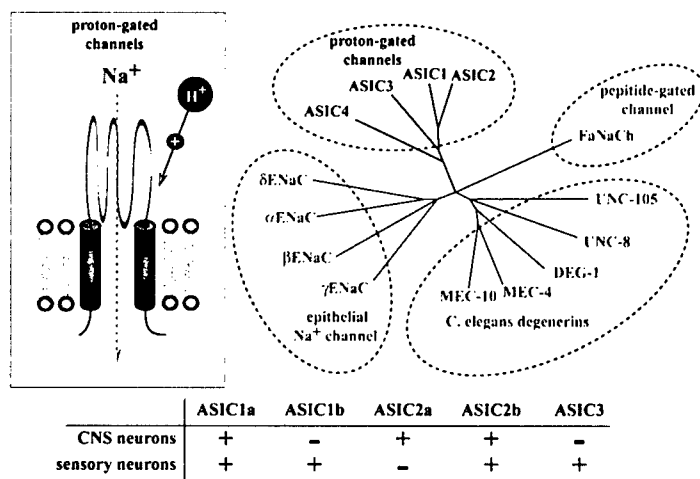


Figure 1. The homologues of "Acid Sensing Ion Channels Channels" and the tissue distribution of ASIC subunits. A, phylogenetic tree showing the epithelial Na^+ channel/ degenerin family of ion channels (reviewed in (35)). αENaC , βENaC , γENaC are the three subunits forming the epithelial amiloride-sensitive Na^+ -channel. δENaC is a related subunit with similar properties as αENaC . δENaC is mainly expressed in testis and the function is unknown. FaNaC is the only ion channel known that is directly activated by a peptide (FMRamide). MEC-10, MEC-4, DEG-1, UNC-8, UNC-105 are degenerins of the nematode *C. elegans*. ASIC1, ASIC2 and ASIC3 are H^+ -gated cation channels. ASIC4 was not functionally expressed as yet (2). B, Different ASIC subunits are differentially distributed in central and in sensory neurons (8, 36).

ASIC1a (35) (also named BNaC2 (14)) and ASIC2b (18) are present in both central neurons and in neurons of the central nervous system. The

ASIC3 (3, 12, 33) and the ASIC1b (ASIC- β (8)) transcripts are mainly present in sensory neurons, while ASIC2a (7) (also named BNC1 (25) or BNaC1 (14)) is only expressed in neurons of the central nervous system. The ASIC channels belong to epithelial Na⁺ channel - degenerin family of ion channels (36), a family of homologous proteins with diverse functions (Figure 1). The amiloride-sensitive Na⁺ channel (27) is involved in transepithelial Na⁺ transport and taste perception. Some of the degenerins of the nematode *C. elegans* (e.g. *mec-4*) are expressed in touch receptors and genetic data suggest that they are involved in mechanosensation (16). They were named degenerins since certain point mutations cause constitutive channel activity and neurodegeneration in the nematode. Interestingly, the corresponding mutations in ASIC subunits also renders the channel constitutively active (7), suggesting that ASIC mutations might possibly also be involved in human neurodegeneration.

H⁺-gated cation channels in sensory neurons

Many painful pathologies such as ischemia and inflammation are associated with a tissue acidosis and there is very convincing evidence that the decreased tissue pH is an important mediator of pain (reviewed in (26)). (i) Lactic acid activates cardiac sympathetic afferents and buffering of the epicardial pH to neutral during experimental myocardial ischemia significantly attenuates the activation of sympathetic afferents (23). However, despite equal epicardial pH, acidic phosphate buffer induced a significantly lower activation of sympathetic afferents and hypercapnia did not cause any activation in the same study. (ii) Injection of local anesthetic solutions is painful unless their acidic pH is neutralized (10). (iii) A subset of nociceptive afferents is excited when the receptive field is exposed to acidic pH (4, 29). The activation threshold lies between pH 6.9 and pH 6.1 for cutaneous nociceptors (29). (iv) Intradermal infusion of acidic solutions provokes the sensation of pain in human (28). The threshold for nociceptor activation reported in those in vivo and in vitro studies matches well the tissue pH reported for many painful pathologies. During myocardial ischemia an epicardial pH of 6.98 (23) was recorded and in tumors the high metabolic activity associated with a limited blood supply can cause a localized drop of the tissue pH to pH 6.6 (15). Severe inflammation and ischemia can be associated with even lower pH (<pH6, reviewed in (26)). While pain caused by many other inflammatory mediators such as histamine or bradykinin is only transient, the pain associated with a tissue acidosis lasts until the tissue pH returns to neutral (28). The sensor for tissue acidosis in sensory neurons might thus be a highly interesting target for the development of novel non-addictive analgesic drugs.

ASIC channels are candidates for the acid sensor in sensory neurons. All but one (ASIC2a) of the known ASIC subunits are expressed there (36). How do the properties of the ASIC channels in sensory neurons fit those expected for an acid sensor involved in nociception? ASIC1a is transiently activated when the extra cellular pH becomes acidic (Figure 2). The channel is mainly permeable for Na⁺, however ASIC1a also has some Ca²⁺ permeability ($pNa^+/pCa^{2+} = 2.5$ (35) or 16 (30)). ASIC1a starts to activate when the extra cellular pH drops to below pH 6.9 (35) (Figure 2), a pH that is attained in ischemic tissues (26). ASIC channels have about 30% sequence homology with the epithelial amiloride-sensitive Na⁺ channel and it is thus not surprising that they are also blocked by the diuretic amiloride and derivatives (35). However the affinity of the ASIC channels for amiloride is much lower ($K_d = 10\mu M$ for ASIC1 (35)) than that of the epithelial Na⁺ channel. Significant block of ASIC channels will not occur with the amiloride concentrations reached during diuresis. Studies with a specific ASIC1a blocking toxin showed that a subset of sensory neurons express native ASIC1a currents (13). ASIC1b, the ASIC1a splice variant has properties quite similar to those of ASIC1a (8, 30), however ASIC1b requires a slightly more acidic pH for activation ($pH_{0.5ASIC1b} = 5.9$ (8) $pH_{0.5ASIC1a} = 6.4$ (30)), is not permeable to Ca²⁺ (8) and is only expressed in sensory neurons (8). The gross tissue pH is likely to decrease slowly during the onset of a tissue acidosis and experiments with human volunteers showed that acidosis induced pain lasts until the pH returns to neutral (28). However both ASIC1a and ASIC1b (ASIC- β) require a rapid drop of the extra cellular pH and desensitize rapidly (8, 30, 35). Common sense would thus rather expect a non-inactivating current that can also be activated when the pH decreases gradually as an acid sensor in sensory neurons. ASIC3 is mainly expressed in sensory neurons and meets those requirements (12, 33). Application of extra cellular acid on ASIC3 expressing cells induces an inward Na⁺ current with a quite peculiar biphasic activation and inactivation kinetics: A rapidly inactivating current is followed by a sustained current (Figure 2). The transient ASIC3 current can only be activated if the extra cellular pH drops rapidly (33). Conversely, the sustained ASIC3 current responds to a slow decrease of pH with a gradual increase of channel activity (33). Both the sensory neuron specific expression and the kinetics seem to make ASIC3 a good candidate for the acid sensor involved in nociception. However, the pH dependence of ASIC3 is quite puzzling. The transient component starts to activate at pH values just below neutral (Figure 2) (12, 30, 33) and reaches half-maximal activity at pH 6.7 (30), a pH dependence matching well the requirements for an acid sensor in nociception. Conversely the sustained component requires very acidic pH (<pH4 for rat ASIC3 (33); <pH6 for human ASIC3 (12)) for activation (Figure 2). While the pH dependence of the sustained human ASIC3 current is one pH unit

shifted from what one would expect for an acid sensor in nociception, the activation threshold of the rat clone is far below physiologically relevant pH values. Furthermore pH values below pH 5.2 provoke decreased nociceptor responses probably due to inactivation of voltage dependent sodium channels (29). There are several possible explanations for this discrepancy. ASIC3 might either be phosphorylated or associated with as for yet unknown interacting proteins *in vivo*. This modification or interaction could shift the pH dependence of the sustained current closer to neutral pH. Clinical pain is caused by a complex synergism of different nociceptor activating or sensitizing compounds. Other components of the "inflammatory soup" might act as co-activators of ASIC3 and shift the pH dependence of the sustained ASIC3 current closer to neutral. However, one possibility that should not be ruled out is, that the sustained ASIC3 current at very acidic pH values is just a property of the ion channel recorded at extreme pH values that does not have physiological relevance.

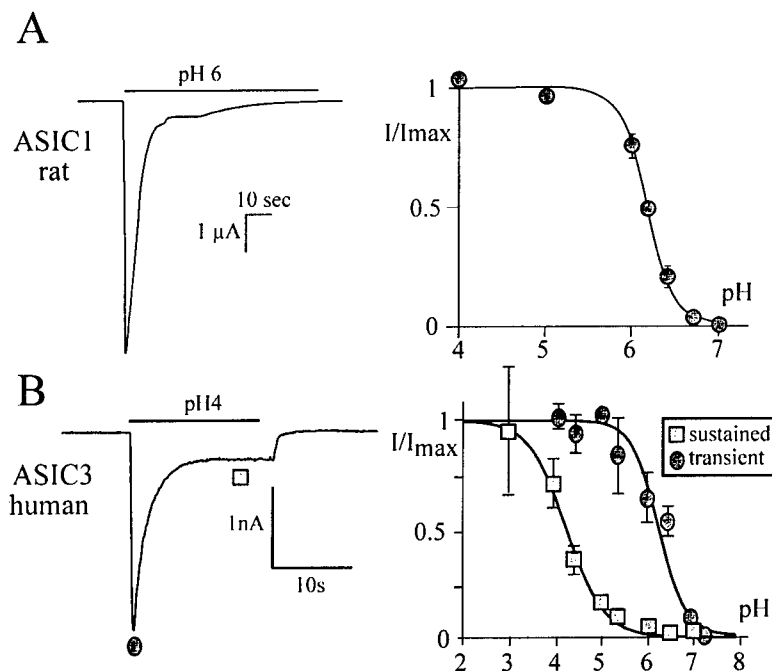


Figure 2. Properties of ASIC1a and ASIC3. A, Properties of rat ASIC1a expressed in *Xenopus laevis* oocytes. B, Properties of human ASIC3 expressed in COS-7 cells. The channels were activated by a rapid pH drop from pH 7.4 to the pH values indicated. The holding potential was -70 mV for A and -60 mV for B. (Modified from (12, 33))

The transient currents of the ASIC subunits expressed in sensory neurons (ASIC1a, ASIC1b, ASIC3) activate when the pH deviates only slightly from neutral pH. Cutaneous nociceptors have activation thresholds of pH 6.9 - pH

6.1 (29), cardiac sympathetic afferents are activated at or just below pH 7 (23) and that is very close to where ASIC1 and the transient ASIC3 channel start to open.

Does the dogma that sustained pain caused by acid requires a sustained cation current really hold? The global tissue pH is likely to vary only relatively slowly during an acidosis. However diffusion is restricted in tissues and the apparently constant tissue pH might mask important local pH fluctuations. The bicarbonate buffering of rapid pH fluctuations within the interstitial space might also be slow or inefficient if the local activity of carbonic anhydrase is low or heterogeneous (reviewed in (9)). Local fluctuations of Ca^{2+} concentration are buffered very inefficiently by Ca^{2+} chelators (22) and the situation might be similar with the buffering of local pH fluctuations by bicarbonate. Transient local acidic pH fluctuations probably occur when acid is released from metabolically active cells, such as contracting myocytes. The dogma that sustained pain requires a sustained current might require a revision. An acid sensor in nociception could either be a non-desensitizing slow cation channel or a cation channel that can be activated during a very rapid acidic transient. The transient ASIC3 current seems to meet this requirement. ASIC3 activates very rapidly ($\tau < 5\text{msec}$) (30) and could thus respond to brief local acidic transients. Furthermore ASIC3 also recovers rapidly from desensitization ($\tau < 0.58\text{ sec}$) (30). The kinetics at physiological relevant temperatures might be even faster than those recorded at room temperature. ASIC channels are not the only H^{+} -gated cation channels known (17). Sensory neurons also express another slowly activating and desensitizing cation channel that can be activated by acidic pH (Figure 3), the capsaicin receptor VR-1 (vanilloide receptor 1) (31). VR-1 is structurally unrelated to the ASIC channels. Heterologous expressed VR-1 is activated by the vanilloide capsaicin, the pungent ingredient of hot chilli peppers, by nocive heat ($> 42^{\circ}\text{C}$), by quite acidic pH ($\text{pH} 0.5 = 5.4$) (31), by endogenous cannabinoide anandamide and by lipxygenase metabolites. VR-1 apparently integrates various pathways involved in nociceptor activation (31). Initially, after the cloning, VR-1 was thought to be the principal heat transducer in sensory nerve endings. However two recently studies with VR-1 knock-out mice demonstrated a surprisingly modest role of VR1 in heat sensation. VR-1 deletion mainly affects inflammatory thermal hyperalgesia (6, 11). There remains the open question regarding the respective role of ASIC channels and vanilloide receptors in acid sensing. VR-1 can be activated by rather acidic pH. However VR-1 activates slowly when exposed to acid and shows profound desensitization in the presence of extra cellular Ca^{2+} (31). Thus, the properties of the heterologous expressed VR-1 do not really match well those expected for an acid sensor responsible for the sustained perception of pain associated with a tissue acidosis. A very promising approach to identify

candidates for H⁺-gated cation channels involved in nociception is the characterization of the acid responses of sensory neurons that innervate a tissue where acid sensing is known to be important. Acidosis seems to play a crucial role in angina pectoris and in the associated afferent modulation of sympathetic activity (23). Sympathetic cardiac afferents are acid sensitive and McCleskeys group demonstrated that the corresponding sensory neurons have, despite their small size, very huge H⁺-gated cation currents (mean 13 nA) with properties almost indistinguishable from those of ASIC3 (5, 30). Those currents are much bigger than the depolarizing currents described in most neurons. A by far sub-maximal activation of ASIC3 might thus be sufficient to depolarize cardiac sensory afferents. The magnitude of the ASIC3 like currents and the low level of the other known acid sensitive cation channel, the capsaicin receptor, suggest that ASIC3 is the acid sensor in sympathetic cardiac afferents (30).

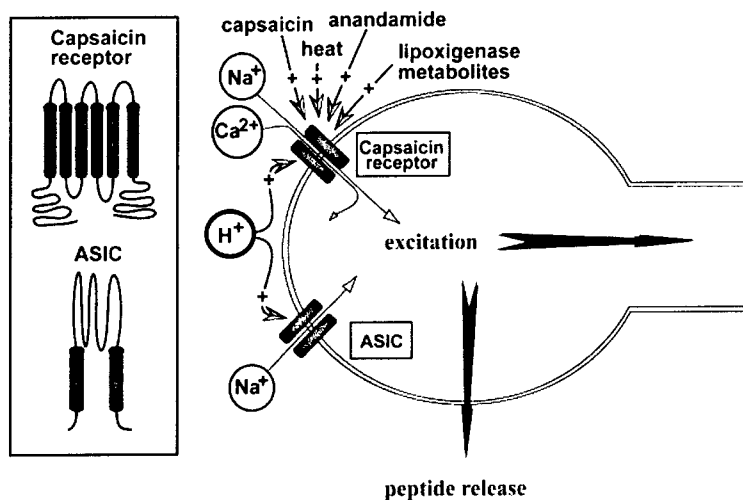


Figure 3. Proton-gated cation channels in sensory neurons. VR-1 integrates various stimuli.

Extra cellular acid potentiates the heat and capsaicin responses of VR-1. High proton concentrations activate VR-1 at 22°C ($pH_{0.5} = 5.4$). At 37°C, pH 6.4 causes some activity of VR-1 (31). Acid evoked VR-1 currents show profound desensitization when extra cellular Ca^{2+} is present (31). Transient ASIC channels in sensory neurons are activated when the extra cellular pH drops to just below neutral ($pH_{0.5} \text{ ASIC3} = 6.7$ at room temperature (30)). Extra cellular acid is the only activator of ASIC channels that was convincingly demonstrated so far.

ASIC channels in the central nervous system

In the central nervous system pH fluctuations occur both during normal brain function (9) and when oxygen supply is limited (21). Many neurons of the central nervous system express proton-gated cation channels and start

firing when the extra cellular pH is decreased rapidly (1, 32). Central neurons express three of the known ASIC subunits (14, 19, 35, 36): ASIC1a, ASIC2a, ASIC2b. ASIC2a is only present in central neurons and absent in the peripheral nervous system (36). The distribution of the different ASIC subunits expressed in the CNS is quite similar if not identical (14, 19, 36). The highest expression levels of the transcripts were detected in the hippocampus, the cerebellum and the cortex. Granular neurons of the cerebellum were shown to express native ASIC1a channels. Those neurons respond to a rapid decrease of the extra cellular pH with a rapidly desensitizing H⁺-gated Na⁺ current that is blocked by a specific ASIC1a blocking toxin (PcTx1) (13). ASIC2a, the subunit specific for the CNS forms as ASIC1a a transient H⁺-gated channel (7). However ASIC2a desensitizes slower than ASIC1a and ASIC2a requires much more acidic pH for activation than ASIC1 (Figure 4). The ASIC2 channel starts to activate when the extra cellular pH drops below pH 5.5. The pH dependence of ASIC2 also raises the question whether such acidic pH occurs in the central nervous system or whether co-activators, associated proteins or post-translational modifications exist that shift the pH dependence closer neutral pH. The pH of the brain remains rather constant during normal brain function, however several mechanisms can produce huge localized pH fluctuations in the CNS. The most important pH fluctuations in the CNS are probably those occurring within or in proximity of the synaptic cleft. Synaptic vesicles use an electrochemical pH gradient to transport neurotransmitters such as glutamate inside. Glutamate containing vesicles are thus acidic. Measurements with a pH-sensitive green fluorescent protein suggest an intravesicular pH of about 5.7 (20). Glutamate containing synaptic vesicles contain a concentrated (>100mM) acidic glutamate buffer. Synaptic clefts are narrow and the release of the vesicle content will create very important acidic pH fluctuations in proximity of the release site. A decrease of the synaptic pH after transmitter release to about pH 6.4 was demonstrated by Miesenbock et al. (20) in their study with the pH-sensitive green fluorescent protein. Additional mechanisms can create pH fluctuations in the CNS. The hydrolysis of neurotransmitters that are esters (ATP, acetylcholine) will also release acid. Given the high local concentrations of those transmitters after release and their rapid hydrolysis, one can assume that the local transient acidic pH fluctuations should be rather important. Rapid pH fluctuations that could activate ASIC1a and possibly also ASIC2a exist in the CNS. However none of the ASIC channel proteins was localized by immunohistochemistry in the CNS as for yet. This will be crucial to demonstrate the physiological relevance of synaptic vesicle release or neurotransmitter hydrolysis associated pH fluctuations for ASIC channel activation.

Brain pH can get very acidic during ischemia (pH 6.43 (21)) or epileptic seizures. Does ASIC channel activation contribute to the associated neuronal death? Both ASIC1a and ASIC2a activate only transiently and desensitize within seconds. Furthermore both ion channels require a rapid pH drop for activation. However the pH decrease during brain ischemia is likely to be gradual. Under those conditions ASIC1a might activate transiently during the initial drop of pH. ASIC1a recovers slowly from desensitization ($\tau=13\text{sec}$) (30) and brain ischemia would thus rather lead to desensitization of ASIC1a than to activation. However, local pH fluctuations associated with neuronal hyperactivity during epileptic seizures might cause activation of ASIC channels. ASIC2a starts to activate at pH 5.5, a global brain pH that will probably not be reached in living mammals. Ischemia would bring the pH closer to the activation threshold of ASIC2a without significantly activating the channel.

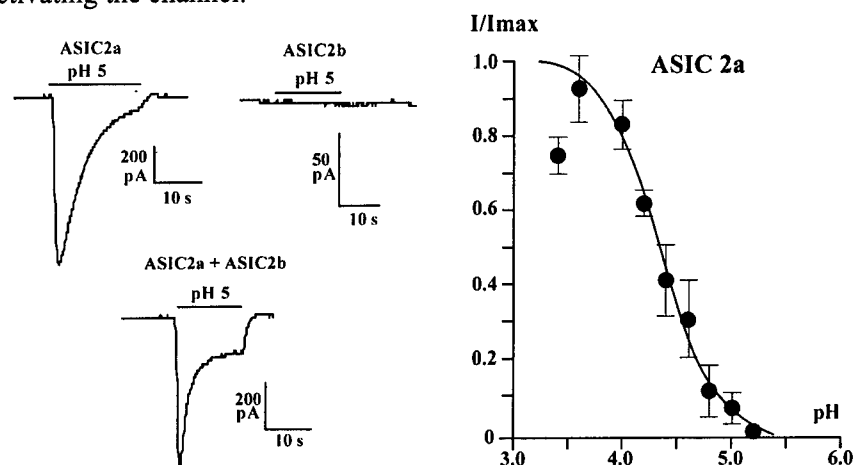


Figure 4. Properties of the homomultimeric and heteromultimeric ASIC2 channels. A, rat ASIC2a, ASIC2b and the heteromultimeric ASIC2a + ASIC2b channel. B, pH dependence of the rat ASIC2a channel. Channels were activated by a rapid drop of the pH from pH 7.3 to to the pH values indicated. The holding potential was -60 mV . (Modified from (7,18,36)).

ASIC subunits can form both homomultimeric but also heteromultimeric assemblies with novel properties. The ASIC2a splice variant ASIC2b is expressed in the central nervous system and the expression pattern matches very closely that of ASIC2a (18). ASIC2b cannot be activated by acidic pH when expressed alone, however co-expression of ASIC2b changes both the ion selectivity and the kinetics of ASIC2a (Figure 4) (18). The heteromultimeric ASIC2a/ASIC2b channel has a biphasic kinetics: a transient rapidly inactivating current is followed by a sustained current. ASIC2a is selective for Na^+ . Co-expression of ASIC2b renders the sustained

current non-selective: K^+ flows as well as Na^+ through the channel. However the heteromultimeric ASIC2a/ASIC2b channel requires pH 5 for activation (18) and it is unlikely that a such acidic sustained pH will be reached even during severe brain ischemia. The known properties of the ASIC channels suggest, that acid activation of ASIC channels does not contribute to neuronal death during ischemia. Static acidic pH is known to causes rather a decrease in neuronal excitability. This is partially due to inhibition of voltage- and ligand-gated cation channels by acidic pH (1).

Quo vadis?

The research on the H^+ -gated cation channels is still in an early stage and there are few answers and many questions. ASIC channels are activated by the simplest but also the most complicated ligand one can imagine - the proton. Virtually every protein is a potential "receptor" for this "ligand" and acidic pH modulates many enzymes and ion channels. This lack of a specific activator renders functional studies on the role of ASIC channels particularly complex. The ASIC currents that are activated within a physiological relevant pH range require rapid pH fluctuations. The physiological role of ASIC channels might not be the sensing of static acidic pH but rather the sensing of local pH fluctuations in the central and peripheral nervous system. However, some of the ASIC channels such as ASIC2 or heteromultimeric channels that contain the ASIC2 subunit require very acidic pH for activation. This imposes the question whether there are other activators of ASIC channels. Certain homologues of the ASIC channels in the nematode *C.elegans*, the degenerins, are involved in mechanotransduction (16). Recently, the group of Michael Welsh reported a somewhat decreased response of rapidly adapting mechanosensitive fibers in ASIC2 knock out mice and suggested that ASIC2 is part of a stretch-activated cation channel (24). However, a stretch activation of ASIC channels or *C.elegans* degenerins was never demonstrated. Extracellular acid is the only known activator of heterologously expressed ASIC channels so far and the proton will be a physiological activator, if pH fluctuation exist in proximity of the ASIC channels, that are important and rapid enough to activate those ion channels. The presence of ASIC channels in the central nervous system opens the highly interesting possibility that the proton might not just be a noxious by-product of metabolic activity but rather the simplest messenger used for cell-to-cell communication in the central nervous system.

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Chapter 20

Structure function relationships of ENaC and its role in sodium handling

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Abstract: The epithelial sodium channel (ENaC) in the apical membrane of polarized epithelial cells is the rate-limiting step for Na entry into the cell; in series with the basolateral Na pump, it allows the vectorial transepithelial transport of Na ions. ENaC is expressed in different epithelia like the distal nephron or colon, and the airways epithelium. In the lung ENaC controls the composition and the amount of pulmonary fluid, whereas in the distal nephron ENaC under the control of aldosterone and vasopressin, is essential to adapt the amount of Na⁺ reabsorbed with the daily sodium intake. Activating mutations of ENaC cause severe disturbances of Na⁺ homeostasis leading to hypertension in human and in mouse models. Functional expression of ENaC in different cell systems allowed the identification of structural domains of the protein that are essential for channel function and/or modulation of channel activity. Site-directed mutations in specific domains of the channel protein lead to channel hyperactivity or channel loss of function. Knowledge about ENaC structure-function relationships opens new opportunities for development of pharmacological tools for controlling ENaC activity, such as channel activators of potential benefit in the treatment of pulmonary edema, or highly potent ENaC blockers with natriuretic effects.

Key words: Epithelial Na⁺ transport, epithelial sodium channel, ENaC, aldosterone, amiloride

INTRODUCTION

Epithelial sodium channels are present in the apical membrane of many salt-reabsorbing epithelia. They are usually identified by their sensitivity to

the diuretic amiloride. Different types of epithelial sodium channels have been described and classified according to their selectivity to Na ions and sensitivity to block by amiloride (10). To date only one of these epithelial sodium channel, the highly selective and amiloride-sensitive epithelial Na⁺ channel ENaC has been identified at the molecular level.

Molecular structure

The availability of a high affinity blocker of ENaC allowed the identification of the primary structure of this channel by function cloning in the *Xenopus* oocyte expression system. ENaC is composed of three homologous subunits α β γ sharing a common membrane topology made of 2 transmembrane domain, a large extracellular domain and two carboxy and amino termini facing the cytoplasmic side (3). The large ectodomain is unique among the members of the gene family to which ENaC belongs and represents more than half of the protein mass. The role of this large ectodomain remains highly hypothetical and could function as receptor for extracellular ligands or could be involved in interactions with the extracellular matrix. Expression of the complementary DNA encoding the three α β and γ subunits in a cell system like *Xenopus* oocytes generate a fully functional channel with the biophysical and pharmacological characteristics of the epithelial sodium channel found in the native tissue. The channel is a heteromultimeric complex composed of 4 subunits, 2 α subunits 1 β and 1 γ subunits arranged pseudosymmetrically around the channel pore (8;9). Expression of the three ENaC subunits is required for maximal expression of ENaC, and homomeric channels resulting from expression of α or β or γ complementary DNAs are inefficiently targeted at the membrane surface and/or result in non functional channels. Channels made of either α and β ENaC subunits or α and γ subunits are functional and reach the membrane surface, but there is a clear preferential assembly of the three α β γ ENaC subunits for channel synthesis when all the ENaC subunits are expressed in the cell.

The epithelial sodium channel ENaC belongs to a gene family that includes a variety of ion channels of different functions. Among the mammalian relatives of ENaC within this gene family, the brain sodium channels activated by protons (ASIC) have recently been identified proposed to play a role in pain and/or mechanotransduction (32).

The human α ENaC gene covers 17 kb on chromosome 12p13. The human β and γ ENaC are located on chromosome 16p in very close proximity. The three ENaC genes share a remarkable degree of conservation in their genomic organization. The human ENaC genes are divided into 13

exons; the two transmembrane regions of ENaC proteins are encoded by parts of exon 2 and exon 13 (23).

The promoters of ENaC genes remain to be precisely identified. For the human and the rat γ ENaC gene, the region within -75 base pairs upstream of the transcription start site including the first exon contains two GC-rich boxes that are sufficient for promoter activity. Analysis of the nucleotide sequence in the region further upstream between -289 and -142 shows 2 imperfect glucocorticoid response elements (GRE) that represent potential transcriptional regulatory elements necessary for ENaC regulation by glucocorticoids. Sequential deletions in this region show that the downstream GRE is sufficient to confer glucocorticoid stimulation and is also able to bind glucocorticoids specifically. An additional GRE motif further upstream (between -300 and -2400) has been identified in the 5' flanking region of human α ENaC. Reporter constructs containing this GRE motif also exhibit glucocorticoid-inducible expression. Until now no mineralocorticoid response element has been identified and the factors that determine the tissue specificity of the corticosteroid regulation of ENaC genes remain to be elucidated.

Physiological role of ENaC

The epithelial sodium channel (ENaC) is a component of the apical membrane of polarized epithelial cells that facilitates Na^+ reabsorption across the epithelium (15). The apical entry of Na^+ ions from the epithelial lumen into the cell occurs through ENaC by electrodiffusion. This apical transport of Na^+ ions is blocked by submicromolar concentrations of amiloride, a pore channel blocker acting from the epithelial lumen. The exit of Na^+ across the basolateral membrane occurs via the Na^+/K^+ ATP-ase. This active transepithelial transport of Na^+ is important to maintain the composition and the volume of the extracellular fluid. For instance in some organs such as the kidney or the colon this transepithelial sodium transport is crucial for the maintenance blood sodium and potassium levels and therefore plays a critical role in the homeostasis of these electrolytes. In the lung or in salivary glands Na^+ transport is certainly not important for the whole body Na^+ homeostasis, but is crucial to maintain constant the composition and the volume of the luminal fluid, the saliva or the alveolar fluid.

ENaC is expressed in a variety of different epithelial tissues such as the kidney, the colon, salivary glands, skin and the lung (6). In the kidney ENaC is expressed mainly in the distal part of the nephron, where under the control of aldosterone and vasopressin it regulates Na^+ reabsorption. The challenge of the distal nephron is to adapt renal Na^+ excretion to the daily Na intake; disturbance of this sodium balance in favor of a Na^+ retention can lead to expansion of the extracellular volume and arterial hypertension. The

pathophysiological role of ENaC has been assessed by human genetic studies and from disruption of ENaC genes in mice. Mutations in ENaC genes leading to hyperactivity has been found in patients with Liddle syndrome, a rare hereditary form of hypertension characterized by an extracellular fluid expansion (13;14;26;29). Loss of function mutations of ENaC genes in patients affected by pseudohypoaldosteronism type-1 cause a renal salt wasting syndrome which can lead to severe dehydration (5).

The airway epithelia can both absorb Na^+ and secrete Cl^- . An amiloride-sensitive electrogenic Na^+ transport is important at birth to clear the liquid that fills the alveoli and the airways of the fetal lung. Messenger RNAs for α β γ ENaC can be detected in the fetal lung around days 15-17 of gestation and expression of ENaC subunits (mainly α and γ) sharply increases in the late fetal and early postnatal life (7;30). During adult life, active Na^+ transport is involved in the maintenance of the composition of the airway surface liquid. The expression of the ENaC subunits along the respiratory epithelium is complex and varies among species. In adult rats and humans the α β γ subunits are highly expressed in small and medium size airways. The α and γ subunits but not the β subunit are expressed more distally in the lung that may well correspond to a localization in the type II alveolar cells. This heterogeneity of the expression of ENaC subunits in the lung epithelia suggests differential regulation of liquid absorption by channels made of various α - β or α - γ subunit compositions. The physiological role of ENaC in lung liquid balance was clearly demonstrated in mice in which the α ENaC gene was inactivated by homologous recombination (16). These α ENaC knock-out mice die soon after birth from respiratory failure due to a severe defect in the clearance of the fetal liquid that fills the lung. The disruption of the β and γ ENaC gene loci results in a slower clearance of the fetal lung liquid at birth which does not severely affect the blood gas parameters. The β or γ knockout mice die later than the α knockout mice but still within the first 50 hours after birth, essentially from a renal Na^+ reabsorption and K^+ secretion defects in the distal nephron leading to severe electrolyte imbalance, namely hyperkalemia (1;25). These studies suggest that in the mouse fetal lung at birth, α ENaC is essential for Na^+ reabsorption. The Na^+ transport in the lungs can sufficiently be maintained by two functional ENaC genes forming α - β or α - γ ENaC channels. However, in contrast to the lung the expression of all three α β and γ ENaC genes is required to provide an efficient Na^+ reabsorption in the kidney and to maintain Na^+ and K^+ balance.

In humans the contribution of α ENaC to the clearance of fetal lung liquid at birth is still under investigation since PHA-1 patients with severe disruption of the α ENaC gene leading to near complete channel loss-of-function do not exhibit respiratory distress syndrome at birth. However

PHA-1 patients having no Na⁺ absorption from the airway surface show a more than two-fold higher liquid volume in airway epithelia than normal individuals (18). Thus there is a clear Na⁺ absorption defect in the airways of PHA-1 patients which does not seem to be limiting at birth for the fetal lung liquid clearance. Differences between species in maturation of the lung, in mucociliary clearance or in ENaC subunit expression in the respiratory epithelium may account for these phenotypic differences between human and mice.

In cystic fibrosis the airway epithelia has an abnormally high rate of Na⁺ reabsorption as reflected by a higher Na⁺ permeability (11). However the molecular and cellular mechanisms of increased Na⁺ absorption in CF patients as well as the role of ENaC in CF is not understood. A number of experimental evidences support functional interactions between ENaC and CFTR, but the nature of these interactions remains controversial. Finally colocalization of ENaC and CFTR in the same epithelial cells remains to be demonstrated.

Functional properties

The epithelial sodium channel ENaC is highly selective for Na⁺ ions and almost impermeant for K⁺ ions. ENaC is constitutively active and does not require electrical stimuli or binding of specific ligands for the channel to open (10). ENaC is blocked by amiloride from the extracellular side with high affinity compared to other non selective cation channels : the inhibitory constant IC₅₀ is below the micromolar range. ENaC blockers such as amiloride or triamterene are used as K⁺ sparing diuretics in the treatment of hypertension to inhibit Na⁺ reabsorption and K⁺ secretion in the distal nephron. The use of amiloride as blocker of ENaC in the lung offers the potential to treat excessive Na reabsorption in the lung of CF patients, and is presently under investigation.

ENaC regulation

Aldosterone and vasopressin are the main hormones regulating Na⁺ transport in the distal nephron and in the colon. In situ immunohistochemistry studies in rodent kidney clearly showed that Na intake and the associated changes in plasma levels of aldosterone affect the intracellular distribution of ENaC subunits(22;24). The increase in plasma aldosterone caused by dietary salt restriction induces a large increase in intracellular abundance of α ENaC and a redistribution of ENaC subunits from the an intracytoplasmic compartment to the apical membrane. Aldosterone acts by binding to a cytosolic receptor and the complex

hormone-receptor translocates to the nucleus to interact with the promoter regions of target genes. These aldosterone-induced or repressed genes mediate the increase in transepithelial Na transport (31).

Antidiuretic hormone such as vasopressin increases ENaC activity and Na reabsorption by binding to a V2 receptor and activation of adenylate cyclase. The ADH effect is mediated by cAMP and activation of PKA; the nature of this phosphorylation has not yet been elucidated. A number of observations suggest that cAMP acts by translocating ENaC from a cytoplasmic pool into the apical membrane, but this issue still remains controversial (19). The stimulation of ENaC activity at the cell surface by ADH is different from that of aldosterone because both effects are synergistic.

Corticosteroids are known to play a role in fetal lung maturation and to favor Na⁺ absorption. In cultured lung and airway epithelia, glucocorticoid hormones increase electrogenic sodium reabsorption. In a cell line derived from lung carcinoma, dexamethasone increases the amiloride-sensitive current by upregulating ENaC subunits (4;21).

Structure-function relationships

Knowledge about the relationships between the structure and the function of ENaC comes from site-directed mutagenesis of the ENaC subunits and the functional analysis of the ENaC mutant channels. The binding site for amiloride is localized in a short segment of the ectodomain of each ENaC subunits preceding the second transmembrane segment (TM2) (28). Since amiloride is a pore channel blocker which plugs the ion permeation pathway for the external side, the pre-M2 segment which contains the amiloride binding site likely forms the outer entrance of the channel pore. In the close vicinity of the amiloride binding site, the extracellular start of the second transmembrane segment constitutes the narrowest part of the channel pore allowing only cations of small size such as Na⁺ or Li⁺ ions to pass through the channel, and larger cations like K⁺ or Rb⁺ ions (17). Thus the outer channel pore appears to narrow from the amiloride binding site down to the selectivity filter; the region forming the inner channel pore beyond the selectivity filter remains to be identified.

The intracytoplasmic amino-terminus of the ENaC subunits contains specific domains involved in the control of channel openings and closings, i.e. channel gating (12). The carboxy-terminus of ENaC subunits have a conserved proline-rich motif involved in interactions with cytoplasmic proteins the cell surface stability of ENaC channel (27). These proline-rich motifs in the β and γ ENaC subunits are mutated in Liddle syndrome patients causing a retention of active ENaC channels at the cell surface and an increase Na⁺ reabsorption. The function of the large ectodomain remains

unknown, but deletion of even relatively small portion of the ectodomain of ENaC subunits usually results in channel loss of function. Whereas the transmembrane part of the ENaC subunits seem to be involved in the formation of the ion permeation pathway, the extracellular and intracellular domains are more likely involved in the regulation of channel activity by factors acting on either side of the membrane.

ENaC regulation has been studied in variety of aldosterone responding tight epithelia such as toad bladder or skin, isolated kidney cortical collecting ducts from rat rabbit or mouse, and different cell lines derived from amphibian bladder or mammalian distal nephron (31). The difficulty of studying ENaC regulation in the lung is the great cellular heterogeneity of the airway and the lung epithelium. However valuable cell lines derived from lung or kidney epithelia are available for the study of ENaC regulation. A cell line derived from human lung epithelial carcinoma A549 cells express the $\alpha \beta \gamma$ ENaC subunits and a channel with the functional characteristics of ENaC (20). Immortalized mouse principal cells of cortical collecting duct (mpkCCDC14 cells) retain a high level of differentiation with epithelial polarity and exhibit amiloride-sensitive sodium transport that respond to aldosterone and vasopressin (2). ENaC subunits are expressed in these cells and patch clamp recordings of apical sodium channels shows the typical functional signature for ENaC. These cell models, together with the widely used *Xenopus* oocyte expression system and gene knockout mouse models by homologous recombination, represent essential tools for the study of the role of ENaC and its regulation at the molecular and cellular levels. The important issues regarding ENaC function and regulation that remain to be addressed include its physiological role in the different tissues where ENaC genes are expressed, and the identification of the different cascades of cellular events induced by hormones leading to ENaC regulation. Together with a better understanding of structure-function relationships of ENaC this research will certainly open new opportunities for development of pharmacological tools for controlling ENaC activity, such as channel activators of potential benefit in the treatment of pulmonary edema, or highly potent ENaC blockers with natriuretic effects.

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Chapter 21

Transepithelial sodium and water transport in the lung

Major player and novel therapeutic target in pulmonary edema

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Abstract: Active transepithelial transport of sodium from the airspaces to the lung interstitium is a primary mechanism driving alveolar fluid clearance. This mechanism depends on sodium uptake by amiloride-sensitive sodium channels on the apical membrane of alveolar type II cells followed by extrusion of sodium on the basolateral surface by the Na-K-ATPase. Injury to the alveolar epithelium can disrupt the integrity of the alveolar barrier or downregulate ion transport pathways thus reducing net alveolar fluid reabsorption, and enhancing the extent of alveolar edema. Endogenous catecholamines upregulate alveolar fluid clearance in several experimental models of acute lung injury, but this upregulation is short-term and often not sufficient to counterbalance alveolar flooding. There is new evidence, however, that pharmacological treatment with beta-adrenergic agonists and/or epithelial growth factors may induce a more sustained stimulation of alveolar fluid reabsorption and in turn facilitate recovery from experimental pulmonary edema. Similar results have been achieved experimentally by gene transfer enhancing the abundance of sodium transporters in the alveolar epithelium. Clinical studies show that impaired alveolar fluid transport mechanisms contribute to the development, severity and outcome of pulmonary edema in humans. Very recent data suggest that mechanisms that augment transepithelial sodium transport and enhance the clearance of alveolar edema may lead to more effective prevention or treatment for pulmonary edema and acute lung injury.

Key words: pulmonary edema, alveolar fluid clearance, transepithelial sodium transport, hypoxia, epithelial sodium channels, Na-K-ATPase, aquaporins, alveolar epithelium, acute lung injury.

INTRODUCTION

Pulmonary edema is a life-threatening condition resulting from an imbalance between forces driving fluid into the airspaces and biological mechanisms for its removal. While for many years Starling forces (hydrostatic and protein osmotic pressures) were thought to play a major role in maintaining the alveolar space free of fluid (111), there is now abundant evidence that active ion transport across the alveolar epithelium creates an osmotic gradient that leads to water reabsorption both during the perinatal period (53,79,35) and in the adult lung (69).

The transporters

Ion transport

Results from several studies *in vitro* and *in vivo* indicate that, in normal conditions, sodium enters the apical membranes of alveolar epithelial cells through amiloride-sensitive cation channels, such as the amiloride-sensitive epithelial sodium channel (ENaC) (78,120,68) and the non-selective cation channel, (58) and is then transported across the basolateral membrane into the interstitium by the ouabain-inhibitable Na-K-ATPase (67,118). Water follows passively, partly through the water channels aquaporins (24,70). Other Na-coupled transporters, such as Na/glucose, Na-K-2Cl symports, Na-phosphate, Na-amino acid and Na-H antiport, have been identified in the lung epithelium but appear to contribute only for a small fraction of the net total transepithelial vectorial sodium and fluid transport. Similarly, the epithelial chloride channels (CFTR) which are also present in the alveolar epithelium do not appear to play a significant role in water transport across the alveolar epithelium in normal conditions (40).

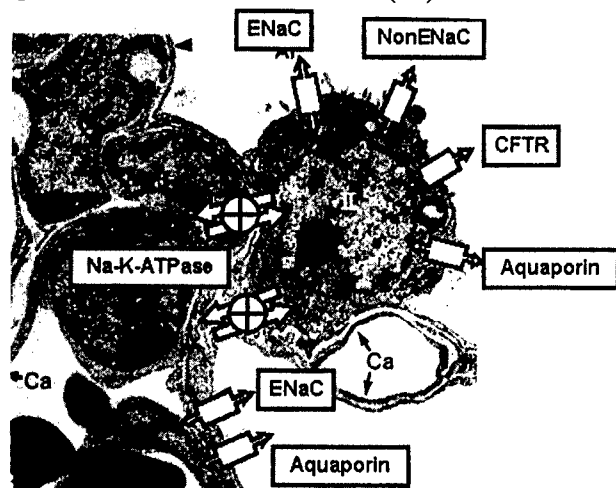


Figure 1. Sodium transport in the lung: the major transporters in the alveolar type II and type I cell.

Initial immunocytochemical evidence *in situ* demonstrated localization of apical sodium channels and Na-K-ATPase in alveolar type II cells, but not in alveolar type I cells (107). From these observations, it was initially inferred that alveolar ion transport was only regulated by type II cells (66) and distal airways epithelial cells (5)). However, preliminary data show that freshly isolated alveolar type I cells express subunits of both Na-K-ATPase and ENaC, suggesting that this cell type might play a role in active vectorial ion and water transport as well (14). Moreover, freshly isolated type I cells exhibited the highest known water permeability of any mammalian cell type, thereby likely explaining the very high water permeability of the lung (25).

Water transport

Water transport across the alveolar epithelial barrier occurs during fluid absorption from the alveolar spaces because a mini-osmotic gradient is created by the vectorial transport of sodium, and perhaps chloride. Water permeability has been measured across several of the major barriers in lung (126). Osmotically-driven water movement across the epithelial barriers in the lung is fast, (33) weakly temperature-dependent, and inhibited by mercurials (19).

Four specialized water transporting proteins, aquaporins (AQP), have been localized in the lung to date: AQP1 in microvascular endothelia and some pneumocytes, AQP3 in basal cells of nasopharynx, trachea and large airways, AQP4 at the basolateral membrane of airway epithelium, and AQP5 at the apical membrane of type I alveolar epithelial cells (127). To define the role of aquaporin water channels in water transport across the various barriers in the intact lung, each of the four lung aquaporins has been deleted in mice by targeted gene disruption (109,127). Deletion of AQP1 or AQP5 produces an approximately 10-fold decrease in osmotically-driven water transport between the airspace and capillary compartments, (4,64) demonstrating the major role of these two aquaporins in regulating the osmotically-driven water movement across the alveolar endothelial and epithelial barriers, respectively. AQP4 deletion had little effect on airspace-to-capillary water permeability (110). Most importantly, however, recent studies show no effect of AQP1, AQP4 or AQP5 deletion, alone or in combination, on isosmolar alveolar fluid clearance and on the development or reabsorption of lung edema following lung injury induced by hyperoxia, thiourea, or acid instillation (109).

Thus, while the precise role of aquaporins in lung physiology remains uncertain, the studies so far demonstrate that active isoosmolar alveolar fluid clearance in the newborn or adult lung does not require lung water channels. Current studies are focused on the possible role of aquaporins in movement of water across the conducting airways of the lung.

Measurement of transepithelial sodium transport

In vitro, patch clamp studies allow the recording of both single channel or whole-cell electrical currents induced by the movement of sodium across the alveolar epithelial cell membrane (62,67). The activity of ion transport pathways can also be assessed in cultured cells by measuring the cellular uptake of radioactive labelled tracer (usually ^{22}Na , ^{86}Rb) (flux measurements) (89,65). In *ex-vivo* isolated lungs, sodium transport has been evaluated by intratracheal instillation of an isoosmotic solution containing a fixed concentration of labelled tracers. The progressive disappearance of ^{22}Na into the perfusate of isolated perfused rat lungs (47) or, alternatively, the progressive increase in labelled albumin concentration in sequential alveolar liquid samples (alveolar fluid clearance measurements), (41,28,70) result from the active transport of salt from the alveolar space into the alveolar interstitium and blood circulation. These techniques have been adapted to measure alveolar fluid clearance also in anesthetized experimental animals and in ventilated patients with pulmonary edema. Finally, another possibility to evaluate the respiratory sodium transport *in vivo* is the measurement of the nasal (or tracheal) potential difference (PD). This PD is generated by the sodium movement across the respiratory epithelium and can be recorded between a reference electrode and an electrode situated in contact with the apical side of the respiratory epithelium under the inferior turbinate in the nose or in the trachea (59,104).

Regulation of the alveolar transepithelial sodium transport

Studies *in vitro* indicate that there is usually parallel, independent regulation of apically localized sodium transport processes and basolaterally located Na-K-ATPase in response to a variety of stimuli, including hormones and growth factors. Intracellular sodium concentration appears to be responsible, in certain cases, for the coupling of the sodium pump and cation channel activities (93,73).

Catecholamine-dependent mechanisms

Beta-adrenergic agonists upregulate alveolar fluid clearance *in vivo* in rats, (23) sheep, (11) dogs, (10) mice, (43,41) as well as in *ex vivo* human lung preparations (94). This effect is mediated, at least in part, by cAMP-dependent mechanisms, (46) is partly inhibited by amiloride, and does not appear to be related to the stimulation of pulmonary blood flow simultaneously induced by these drugs (11).

Beta-adrenergic agonists are effective when delivered either intravenously or directly into the distal airspace of the lung (11,121). Although early

studies indicated that the primary stimulating effect was mediated by beta-2 receptors, recent work shows that beta-1 stimulation is also effective in upregulating alveolar fluid clearance (77,97). Consistent with these data, both beta-1 and beta-2 receptors are present on the apical and the basolateral surface of the alveolar epithelium (18).

It is important to note, however, that, in normal conditions, endogenous catecholamines do not play a major role in maintaining baseline transepithelial sodium transport as demonstrated by normal alveolar fluid clearance in adrenalectomized animals, (42) and unaltered basal alveolar clearance after beta-blockade in animals, (11,56) and in the ex-vivo human lung (95).

Several possible mechanisms underlying the catecholamine-dependent regulation of active sodium absorption across the alveolar epithelium have been proposed, and virtually every step from the gene expression to membrane insertion of the transporters has been implicated in the regulation of vectorial salt and water transport across the distal airspaces of the lung (Table 1).

There is increasing evidence that in the airways, cystic fibrosis transmembrane conductance regulator protein (CFTR) can regulate ENaC function (114). Indeed, in the presence of beta-agonists or cAMP, indirect stimulation of the transcellular sodium movement by stimulation of an apical chloride conductance has been reported by different groups (57,91,55). These important results suggest that a critical factor in upregulating fluid clearance might be chloride, rather than just transepithelial sodium

Table 1. Proposed mechanisms implicated in the regulation of active sodium transport across the alveolar epithelium.

Transcription	Increased ENaC gene expression	(68,74)
	Increased Sodium Pump gene expression	
Translation	Increased ENaC protein synthesis	(52)
	Increased Sodium Pump protein synthesis	
Intracellular trafficking	Increased ENaC Trafficking	(108)
	Increased Sodium Pump Trafficking	(101)
Membrane insertion	Increased Sodium Pump abundance	(68)
	Increased ENaC abundance	(67,139)
Altered transporter configuration	Phosphorylation or the ENaC or Sodium Pump	(68,74)
	Covalent or allosteric modifications	
Transporter internalization and recycling	(?)	(12)
Transporter degradation	Ubiquitination	(112)

absorption. Consistent with this possibility, preliminary data suggest that beta2-adrenergic stimulation of the alveolar fluid clearance is absent in mutant mice deficient for the CFTR channel, and in *in situ* lung preparations of wild type mice (40) or ex-vivo human lungs instilled with the chloride channel inhibitor, glibenclamide.

Catecholamine-independent mechanisms

Growth Factors

In addition to the well studied effects of β -adrenergic agonists, several catecholamine-independent pathways can increase the rate of alveolar fluid clearance. Incubation of isolated alveolar type II cells with epidermal growth factor (EGF) for 24-48 hours increases their capacity to transport sodium (15) and upregulates alveolar fluid clearance in rats (119). Keratinocyte growth factor (KGF), an important alveolar epithelial type II cell mitogen, induces a similar effect primarily by stimulating alveolar type II cell proliferation (129,49). Transforming growth factor-alpha (TGF- α) can increase alveolar fluid clearance acutely in anesthetized, ventilated rats by a cAMP-independent mechanism (37).

Vasoactive Agents and Hormones

Dobutamine markedly upregulates alveolar epithelial fluid clearance in rats by stimulating beta-2 receptors, (121) whereas dopamine upregulates alveolar fluid transport by stimulating the dopaminergic receptor D1 (101,9). Glucocorticoids and thyroid hormone increase respiratory transepithelial sodium transport during the foetal and perinatal period in several animal species (8). Recent observations indicate that these hormones may also upregulate sodium transport and fluid clearance in adult animals (78,120,36). Finally, insulin and estrogens have also been shown to increase sodium transport across cultured ATII cells (122,117). However, it is not clear whether the concentrations needed to increase sodium transport *in vitro* also occur in the lung interstitium *in vivo*.

Strategies to stimulate sodium and water transport in the normal lung

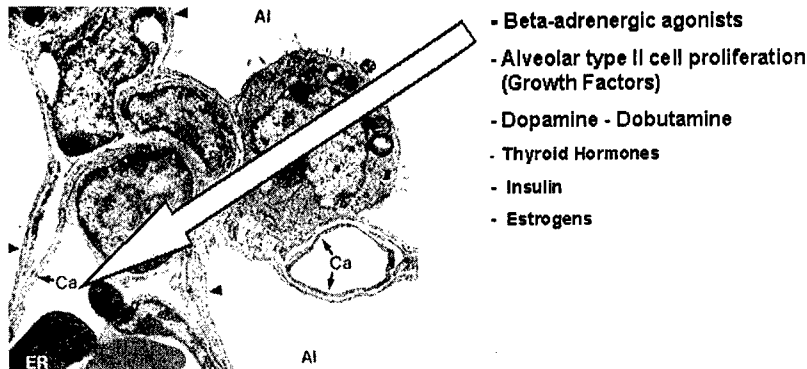


Figure 2. Strategies to stimulate sodium and water transport in the normal lung

RESPIRATORY TRANSEPITHELIAL SODIUM AND WATER TRANSPORT IN EXPERIMENTAL ACUTE LUNG INJURY

It is now well established the transepithelial sodium transport plays a major role in the clearance of fluid from the airspace not only in normal conditions but also during experimental lung injury. First, α ENaC deficient mice die shortly after birth from lung edema (53). Transgenic expression of α ENaC in α ENaC deficient mice (α ENaC $(-/-)$ Tg) rescues the lethal pulmonary phenotype, (54) but results in an infraclinical defect of transepithelial sodium transport. This defect is associated with roughly a 50% lower rate of alveolar fluid clearance and a significantly larger hypoxia-, hyperoxia-, and thiourea-induced pulmonary edema (28,26). Second, instillation of phenamil (an irreversible blocker of epithelial sodium channels) into the lung of rats exposed to hyperoxia resulted in a significant increase of the extravascular lung fluid volume (138). Third, systemic administration of amiloride facilitates the development of thiourea-induced pulmonary edema in mice (Sartori, unpublished data). Finally, pharmacological stimulation of the respiratory epithelial sodium transport facilitates the recovery from pulmonary edema in several experimental models of acute lung injury (see below).

Upregulation of alveolar fluid transport in acute lung injury

Animal models of acute lung injury have been used to assess the mechanisms regulating transepithelial sodium and water transport under these pathological conditions. In many, but not all, of these *in vitro* and *in vivo* models, sodium and fluid transport is upregulated. Indeed, lung injury after hyperoxia, (80,139,61,99) thiourea, (140) hemorrhagic shock, (83) septic shock, (86) as well as neurogenic pulmonary edema (60) are all associated with upregulated transepithelial sodium and water transport.

The major underlying mechanism stimulating this transport appears to be a stress-induced release of endogenous catecholamines, (83,60) possibly in association with a yet to be confirmed direct stretch-sensitive mechanism in the alveolar wall to detect volume overload (133). Consistent with this concept, adrenalectomy impairs alveolar fluid clearance and facilitates thiourea-induced pulmonary edema (Sartori, unpublished data).

In addition to catecholamine-dependent mechanisms, other pathways may stimulate fluid transport across the alveolar epithelial barrier in the presence of lung injury. For example, stimulatory effect of endotoxin (45) may depend on the release of cytokines, since a monoclonal antibody against TNF- α inhibited the increase in alveolar fluid clearance that occurred 24 hours after development of gram negative bacterial pneumonia in the rat lung (92).

Proliferation of alveolar epithelial type II cells provides another catecholamine-independent mechanism for accelerating fluid transport across the injured alveolar epithelial barrier. Indeed, the marked stimulation of alveolar fluid clearance observed in the sub-acute phase of bleomycin-induced acute lung injury in rats appears to depend mostly on the extensive proliferation of alveolar epithelial type II cells (34).

Downregulation of alveolar fluid transport in acute lung injury

There exist few models of acute lung injury in which the transepithelial sodium transport appears to be down-regulated.

During ventilator associated lung injury in rats, the ability of the lung to clear edema is impaired. The mechanisms involved are not clear but may include an increase in lung endothelial and epithelial paracellular permeability (38) and/or downregulation of alveolar transport proteins (63).

Release of nitric oxide by activated alveolar macrophages and/or respiratory epithelial cells (102) inhibits sodium transport in lung injury models associated with airway inflammation (51,71). Interestingly, in rats, the downregulation of alveolar fluid clearance associated with systemic hypotension is reversed by the administration of nitric oxide inhibitors (84).

Reactive Oxygen Species (ROS) have also been shown to downregulate *in vivo* alveolar fluid clearance in a rat model of lung injury induced by ischemia-reperfusion (75,96). Finally, very recently it was reported that inhibition of the caspase cascade attenuated the inhibitory effect of *Pseudomonas* pneumonia on alveolar fluid clearance (Guéry, personal communication), suggesting that exaggerated apoptosis may contribute to decreased respiratory sodium and fluid transport in some conditions.

Hypoxia and respiratory transepithelial sodium transport

Hypoxia is a condition frequently observed in physiological (high altitude) or pathological conditions (for example during hypoventilation, pulmonary edema, obstructive lung disease) associated with lung injury. Although the respiratory epithelium is directly exposed to hypoxia little information on the effects of low oxygen tension on alveolar epithelial cell function was available so far (21). Recently, evidence both *in vitro* and *in vivo* suggests that hypoxia significantly affects the transepithelial sodium transport in the lung by a number of different mechanisms.

In vitro, hypoxia inhibits sodium transport in alveolar type II cells (89,135,52,65) by impairing both amiloride-sensitive and -insensitive sodium pathways. After acute exposure to hypoxia (15-30min), transepithelial sodium transport appears to be inhibited, probably by transporter inactivation (135). A more prolonged (4-12 hours) exposure downregulates the **gene** expression of the 3 subunits of the ENaC and of the $\alpha 1$ and $\beta 1$ subunits of the Na-K-ATPase (88,89). This downregulation is associated with a decreased protein synthesis of ENaC and sodium pump, it parallels the inhibition of the ENaC transport and Na-K-ATPase hydrolytic activity and is fully reversed by reoxygenation. Whether other mechanisms, such as impaired mRNA translation, altered intracellular trafficking or abnormal degradation or internalization of these transporters, may also contribute to hypoxia-induced inhibition of sodium transport is currently under investigation.

The mechanisms underlying the inhibitory effect of hypoxia on gene expression and protein synthesis are unknown. At low oxygen concentrations decreased levels of cellular ATP have been shown to contribute, at least in part, to the reduction of transepithelial sodium transport (89). However, other mechanisms directly dependent on the level of oxygen, and not on metabolic limitations, may also play an important role (65). Indeed, the alveolar epithelium is able to maintain a normal energy status after prolonged (24-48h) exposure to hypoxia, (116) and alveolar type II survive at least 24h without detectable cellular damage and have a cellular ATP content close to that in normoxic conditions (81). Recent data in fetal distal lung epithelial cells suggest that reactive oxygen species (ROS) (52)

and nuclear factor-kB (NF-KB) (90) may contribute to hypoxia-induced effects on respiratory sodium transport.

In vivo, exposure to prolonged hypoxia decreases the nasal potential difference in rats (123) and in humans (103). Furthermore, it decreases the alveolar fluid clearance in rats (115) and in ex-vivo human lungs (95). Whether this hypoxia-induced inhibition of the sodium transport contributes to the pathogenesis of pulmonary edema *in vivo* is not known. It is important to note, indeed, that hypoxia augments the number of beta-adrenergic receptors in the lung (13) and stimulates the release of endogenous catecholamines, two mechanisms that may counterbalance, at least in part, its negative effects on transepithelial sodium transport and alveolar fluid clearance.

PHARMACOLOGIC STIMULATION OF ALVEOLAR FLUID CLEARANCE IN EXPERIMENTAL ACUTE LUNG INJURY

The severity and outcome of acute lung injury depends, at least in part, on the balance between the extent of vascular endothelial and alveolar epithelial damage and the effectiveness of endothelial and epithelial repairing mechanisms. The alveolar epithelium is remarkably resistant to injury, particularly compared to the adjacent lung endothelium (87). Even when mild to moderate alveolar epithelial injury occurs, the capacity of the alveolar epithelium to transport salt and water is often preserved (134). In addition, as discussed above, several mechanisms may upregulate the fluid transport capacity of the distal pulmonary epithelium during lung injury. However, this upregulation may not be sufficient to counterbalance the alveolar flooding, and pulmonary edema may develop.

During ALI/ARDS, the ability to remove alveolar fluid rapidly (related to preserved alveolar epithelial function) is associated with improved oxygenation, a shorter duration of mechanical ventilation, and an increased likelihood of survival (132,72,125). Therefore, efforts to enhance the reabsorptive capacity of the alveolar epithelium may be as important as those aimed to attenuate the lung endothelial and alveolar epithelial injuries.

Beta-adrenergic agonists

Beta-agonists are attractive as therapeutic agents because they are already in wide clinical use and have minimal side effects, even in critically ill patients.

There is growing evidence that, in the presence of lung injury, alveolar fluid clearance can be further stimulated with exogenous beta-adrenergic therapy (61,44,100).

In experimental hydrostatic pulmonary edema, beta-agonists accelerate the resolution of alveolar edema (39,17). Beta2-adrenergic agonists augment the rate of alveolar epithelial fluid transport in rats in the presence of moderate lung injury from hyperoxia, (44,61) and restore the rat lung ability to clear edema in ventilator-associated lung injury (100).

Under some circumstances, however, the epithelium may not respond to beta2-agonists because of extensive epithelial injury and loss of alveolar type II cells, or because of suppression of the normal ability of type II cells to increase alveolar fluid clearance by the inflammatory environment. For example, following prolonged hemorrhagic shock in rats, reactive oxygen species inhibited the response of the alveolar epithelium to beta2-agonist stimulation (75). In addition, down-regulation of beta- receptors and, in turn, diminishing therapeutic effect of beta2-agonists over time (29) need to be considered. However, recently published studies demonstrated no evidence of down-regulation when high doses of epinephrine were delivered to rats over a period of four hours (20).

Growth factors

Since acute injury to alveolar epithelial type I cells frequently causes denudation of the alveolar epithelium, (76,3) re-epithelialization of the alveolar barrier may represent an additional approach to hasten the resolution of acute lung injury and the acute respiratory distress syndrome (131). The provision of a new epithelial barrier with alveolar type II cells may have beneficial effects in addition to restoration of the air-liquid interface.

Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are major mitogens for alveolar epithelial type II cells. Intratracheal pretreatment of rodents with KGF prior to induction of lung injury with radiation, (137) thiourea, (49,50,27) hyperoxia (16,6) or acid instillation, (136) decreased the severity of lung injury and overall mortality.

This effect required high doses of the growth factor delivered by the intratracheal route and the maximal effect occurred only after 48 to 72 hours. After 48 hours, KGF produces a sustained upregulation of alveolar fluid clearance lasting for several days (129) which can be further enhanced by the addition of a beta2-agonist (128). Taken together, these data suggest the possibility to provide both short term (beta-agonists) and longer term (growth factors) up-regulation of the alveolar fluid transport that might hasten the resolution of clinical pulmonary edema.

Gene Therapy

Several experimental studies have demonstrated that alveolar edema clearance correlates with Na-K-ATPase activity in both normal and acutely injured animal lungs (98,9,11,61). Thus, another potential approach to increase sodium transport and alveolar fluid reabsorption would be to overexpress the Na-K-ATPase gene in the alveolar epithelium.

Consistent with this hypothesis, overexpression (with adenoviral gene transfer) of the beta-1 or the alpha2 subunit increased sodium pump expression and function in the adult rat lung, (32,2) and was associated with increased survival in rats exposed for 64 hours to hyperoxia (31), or in mice exposed to thiourea (113). Surprisingly, for unknown reasons, the overexpression of the catalytic (alpha1) subunit of the pump did not induce a similar effect (32).

Gene transfer technology (30) or transgenic overexpression (42) have also been used to overexpress the beta2-adrenergic receptor. In rats and mice, such overexpression stimulates the liquid clearance in the normal lung by increasing its sensitivity to endogenous catecholamines (30,42). However, preliminary data suggests that the beta2-agonist-stimulated alveolar fluid clearance is comparable in these animals and their wild-type littermates.

Further studies are needed to assess the potential benefits of gene therapy to the alveolar epithelium in the treatment and prevention of lung edema.

ALVEOLAR EPITHELIAL INJURY AND SODIUM TRANSPORT FUNCTION IN HUMAN FORMS OF PULMONARY EDEMA

Biological markers of lung injury

In the attempt to detect potential biological markers of both endothelial and/or epithelial lung injury, (85,76) several studies have compared the concentrations of different biologically active substances in samples of undiluted edema fluid collected from mechanically ventilated patients with ALI/ARDS to those of appropriated control samples collected from ventilated patients with hydrostatic pulmonary edema. In patients with acute lung injury, the edema fluid concentrations of substances such as ICAM-1, (22) hepatocyte growth factor (HGF), (124) and transforming growth factor-alpha (TGF- α) are higher than the corresponding plasma concentrations and significantly increased compared to control subjects. These findings suggest that these substances are released directly into the alveolar space when the lungs are injured, and may be used as a marker of injury of the alveolar barrier. Furthermore, preliminary data indicate that high pulmonary edema

fluid levels of ICAM-1 and HGF may also be associated with impaired alveolar fluid clearance and prolonged duration of assisted ventilation in patients with ALI/ARDS, (22) suggesting a possible role of these markers as predictors of clinical outcome.

Table 2. Potential clinical applications of treatments designed to enhance the resolution of alveolar edema

Aerosolized Beta-Agonists		
Salmeterol	High-altitude pulmonary edema	(105)
Salmeterol	Hydrostatic pulmonary edema-experimental	(17,39)
Isoproterenol/Terbutaline	Acute lung injury from hyperoxia	(44, 61, 100)
Isoproterenol/Terbutaline	Ventilator-associated lung injury	(100)
Vasoactive Agents		
Dopamine	Acute lung injury from hyperoxia	(9), (101)
Epinephrine		(20)
Growth Factors		
Keratinocyte Growth Factor (intra-tracheal)	Radiation	(137)
	Thiourea	(49)
	Bleomycin	(137)
	Hyperoxia	(82), (16), (6)
	Acid instillation	(136)
Glucocorticoids		
Solumedrol/Dexamethasone	Acute lung injury	(36)
Gene Therapy		
Na-K-ATPase β 1-subunit overexpression	Hyperoxia	(32)
Na-K-ATPase α 2-subunit overexpression	Hyperoxia	(2)

The clinical significance of these findings, and the clinical relevance of recently discovered increased edema fluid concentrations of several other substances (such as leukotriene D₄, leukotriene B₄, substance P, interleukin-8, interleukin-6, FAS/FAS-L, (130) and HTI-56 (a new integral membrane antigen specific of alveolar epithelial type I cells) (76) are still unclear. Their role in injuring or repairing the alveolar epithelial barrier in patients with acute lung injury, their utility as possible markers of respiratory epithelial injury, and their potential contribution in the development of multi-organ failure secondary to ARDS are being currently studied (85).

Genetic alteration of the sodium transport

Newborns infants with either transient tachypnea (48) or neonatal distress syndrome (7) have a lower nasal potential difference, a marker of transepithelial sodium transport across the epithelium in the distal airways. In these patients, the nasal PD was poorly inhibited by amiloride. These results suggest that an impairment of sodium absorption across the respiratory epithelia of very premature infants may be one factor contributing to the pathogenesis of neonatal respiratory distress syndrome.

We hypothesized that a similar impairment may also augment the susceptibility to pulmonary edema in adults. High-altitude pulmonary edema (HAPE) is a paradigm of pulmonary edema because it occurs in predisposed, but otherwise healthy, subjects thus making it possible to study the underlying mechanism in the absence of confounding factors (i.e drugs or cardiac dysfunction) (106). We found that, compared to HAPE-resistant subjects, patient prone to develop HAPE have impaired sodium transport when exposed to high altitude (103). Second, and even more importantly, it demonstrated that, already at low altitude, the nasal PD was roughly 30 percent lower in HAPE-prone than in HAPE-resistant subjects. These exciting preliminary findings provided for the very first time the possibility of a genetic impairment of the respiratory transepithelial sodium and water transport in a human form of pulmonary edema (104).

Pharmacological stimulation of alveolar fluid clearance in clinical medicine

An important question is to determine whether pharmacological stimulation of the alveolar sodium transport accelerates the clearance of pulmonary edema, not only in experimental animal models, but also in humans. Two lines of evidence suggest that this is indeed the case.

First, recent clinical data show that high concentrations of albuterol ($>10^{-6}$ M) are present in the undiluted edema fluid of mechanically ventilated patients with ARDS after a single inhalation by standard aerosolization (1). These results indicate that aerosolization of a beta-agonist are sufficient to deliver the drug at therapeutic concentrations in the distal airspaces of the lung.

Second and even more excitingly, in a very recent preliminary study, inhalation of the lipid soluble beta2-agonist, salmeterol, prevented the development of high altitude pulmonary edema in predisposed subjects, suggesting that the impaired respiratory transepithelial fluid transport in these subjects (104) may be restored by the administration of a beta-adrenergic agonist (105).

SUMMARY

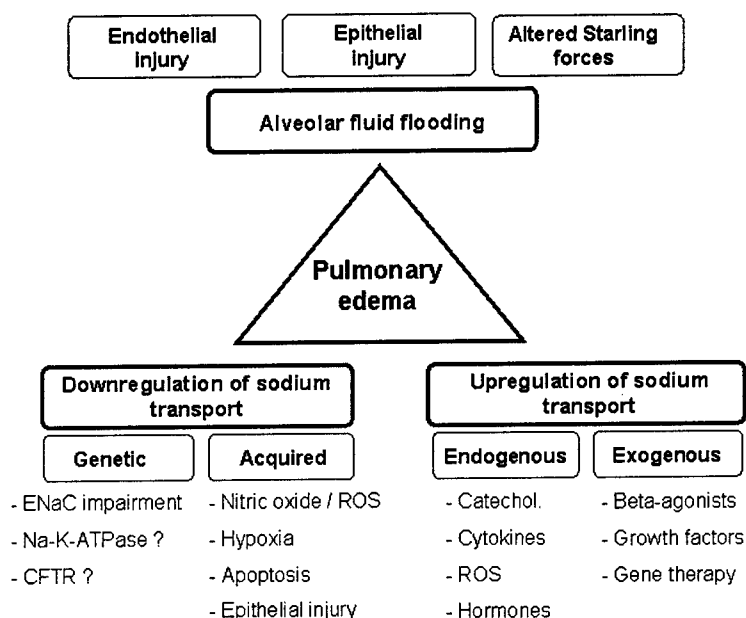


Figure 3. Pathophysiological summary.

Pulmonary edema can be viewed as a perturbation of the balance between the forces driving water into the airspace and the biological mechanisms for its removal. Injury of the lung endothelium and/or epithelium and an alteration of the balance of the Starling forces may result in pulmonary edema with alveolar flooding. The data summarized in this review, demonstrate that active respiratory transepithelial sodium transport plays an important role in generating the driving force that facilitates the clearance of fluid from the alveolar space into the interstitium and the vascular space, and in turn, in the pathogenesis of pulmonary edema.

In acute lung injury, multiple factors may alter the respiratory transepithelial sodium. In some conditions, this transport may be downregulated (for example by the release of inflammatory molecules), whereas in others, stress-related release of endogenous substances such as catecholamines, cytokines, hormones and growth factors may upregulate the sodium transport and stimulate the alveolar fluid clearance. Endogenous stimulation of this transport may, however, not be sufficient to compensate for the alveolar flooding and prevent pulmonary edema. Therefore, exogenous stimulation of the respiratory transepithelial sodium and water transport has been tried in experimental models of lung injury. So far, beta-adrenergic agonists, epithelial growth factors, and adeno-viral mediated

overexpression of epithelial sodium transporters have given encouraging results.

Before these strategies may be implemented into clinical practice, however, important issues need to be resolved. For example, for gene therapy, viral vector-induced inflammation in an already damaged lung, and the possibility of the development of a secondary systemic injury may limit its beneficial effects. Moreover, the benefit of transfection once injury has occurred have not yet been established, and the optimal delivery method remains to be determined. While it is possible that transfection may be of little use in the acute setting, it may prove beneficial in more long-term, volume overload states such as congestive heart failure, or as pretreatment of donor lungs to prevent reperfusion pulmonary edema. With regard to the clinical use of growth factors stimulating alveolar type II cell proliferation, there are concerns about their long-term efficiency, and the possible induction of epithelial dysplasia. At the time of this writing, pharmacological, beta-adrenergic stimulation of transepithelial sodium transport is most close to its clinical introduction. What we now need, is the demonstration that upregulation of alveolar ion and fluid transport will either prevent pulmonary edema or enhance the resolution of alveolar edema with an improvement in clinical outcomes. Preliminary data suggest that this may indeed be the case. We suggest that the pursuit of this important avenue of research will generate important new insight into the regulation pulmonary fluid homeostasis, and will have major implications for both the treatment and prevention of pulmonary edema.

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Chapter 22

Is ventilatory acclimatization to hypoxia a phenomenon that arises through mechanisms that have an intrinsic role in the regulation of ventilation at sea level?

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Abstract: The purpose of this article is to set out the hypothesis that arterial Po_2 may play a significant role in the regulation of breathing at sea level. The following points are made: 1) Although CO_2 is clearly the dominant feedback signal in the acute setting, there is evidence, particularly clinical observation, that the ventilatory response to CO_2 may adapt. 2) Although the ventilatory response to an acute variation in alveolar Po_2 around sea-level values is feeble, studies at altitude have shown that over longer-time periods alveolar Po_2 is a more powerful regulator of ventilation. 3) Recent evidence suggests that mechanisms associated with ventilatory acclimatization to hypoxia are active at sea-level values for Po_2 , and indeed affect the acute ventilatory response to hypoxia. 4) While most evidence suggests that the peripheral and central chemoreflexes are independent and additive in their contributions to ventilation, experiments over longer durations suggest that peripheral chemoreceptor afferents may play an important role in regulating central chemoreflex sensitivity to CO_2 . This is potentially an important mechanism by which oxygen can alter the acute chemoreflex responses to CO_2 . In conclusion, the mechanisms underlying ventilatory acclimatization to hypoxia may have an important role in regulating the respiratory system at sea level.

Key words: human; altitude, peripheral chemoreflex, central chemoreflex, chemoreceptors, breathing

INTRODUCTION

Our understanding of the biology of oxygen homeostasis is developing rapidly. In particular, we have seen an explosion in knowledge relating to the control of gene expression by hypoxia. Over the last decade, these advances include the discovery of hypoxia-inducible factor and an appreciation of the wide range of genes that are subject to regulation by this mechanism (23). Following such progress on the molecular front, it is an appropriate time for more integrative physiologists to take stock, and reflect on how these advances affect the particular questions they are pursuing.

What are the main questions within respiratory control? We have, and have had for a long time, a reasonable understanding of the peripheral and central chemoreflexes regulating breathing. We are aware of the major stimuli and the general properties of these reflexes. We know of ventilatory acclimatization to altitude, and have a reasonable description of the phenomenon. Clearly there are matters relating to the mechanisms underlying these processes that we do not yet understand. Notable amongst these are the nature of the oxygen sensing process, central chemoreception generally and the precise nature of respiratory rhythm generation. However, these are matters of detail, important detail, but detail nonetheless within the framework of a control system that we do understand. Or, do we?

One very fundamental question is how do we end up with the control system that we have? For example, how do we end up with a chemoreflex sensitivity to CO_2 that is capable of providing a vigorous defence against asphyxia, but still remains below that which would generate feedback instability within the chemoreflex loop? How do we end up with an exercise response that is isocapnic at work rates below the anaerobic threshold? Indeed how does respiratory control adapt in relation to growth and development, from the relatively stiff lungs and compliant ribcage of the young child through to the relatively compliant lungs and stiff ribcage of the adult? In relation to morphology, the field of developmental biology is extremely well established. However, related questions exist with respect to physiological development. The purpose of this article is to consider the general question of how may the respiratory control system itself be regulated and the more specific question of whether oxygen has any role to play in this process.

Carbon dioxide and the control of breathing

A century ago, Haldane and Priestley (12) in their landmark paper identified the PCO_2 of the alveolar gas as the link between ventilation (\dot{V}_E) and metabolism. This belief of the primacy of CO_2 over O_2 in the feedback regulation of \dot{V}_E has largely persisted through into modern physiology. For

example, West (27) writes, "The most important factor in the control of ventilation under normal conditions is the PCO_2 of the arterial blood." For data from our laboratory, we would estimate that \dot{V}_E is ~30 fold more sensitive to variations in arterial PCO_2 around normal values at sea level than it is to variations in arterial PO_2 .

Despite the obvious power of the feedback response to CO_2 in the acute setting, an important question remains as to whether the CO_2 response adapts. By way of analogy, it is very clear in the acute setting that arterial baroreceptors form the major feedback loop regulating blood pressure, and intact renal function is of no real importance. However, over longer time periods, it is clear that the kidneys play a major role in determining blood pressure, with the powerful baroreflex resetting to operate around a blood pressure which is determined through renal function. Does the CO_2 response adapt and reset in an analogous manner? Studies of the longer-term responses to CO_2 inhalation are far fewer in number than are studies of the acute responses. They have been reviewed by Dempsey and Forster (5) who concluded, "Pulmonary and alveolar ventilation remains above control and at or above levels during acute CO_2 breathing throughout 5-42 days of breathing 1-4% CO_2 ." At first sight, this seems to suggest that the CO_2 response does not adapt, at least in the medium term. However, these experiments are difficult to interpret because the feedback loop between \dot{V}_E and alveolar PCO_2 remains intact. This minimises the variation in response over time; as \dot{V}_E goes up, so alveolar PCO_2 goes down, and *vice-versa*. Quantitatively, the importance of this point can be seen from the fact that, acutely, a 3% (21 Torr) rise in inspired CO_2 might elevate alveolar PCO_2 by 4 Torr, but if alveolar ventilation were to return to normal, the rise in alveolar PCO_2 would have to be 21 Torr. Thus it is not completely clear from these experiments that the \dot{V}_E response to alterations in CO_2 does not adapt over time.

It is difficult to achieve longer exposures to elevated levels of CO_2 in healthy human volunteers. However, many patients suffer lung disease and the pathophysiology associated with this certainly casts doubt upon the notion that the respiratory output, or effort, in response to CO_2 cannot adapt over the long term. At one extreme there are patients, the pink puffers, who make an intense respiratory effort in response to their lung disease, despite the fact that their arterial PCO_2 may be normal, or even low (2). But at the other extreme, there are the blue bloaters, who can have extremely high values for arterial PCO_2 , but make little respiratory effort.

Oxygen and the control of breathing

As mentioned above, Haldane and Priestley (12) found that the respiratory response to variations in PO_2 around normal values was feeble

compared with PCO_2 . Thus, in order for O_2 to play any significant role in the regulation of breathing, its effects over longer time periods would need to be far more substantial. Much of the evidence in support of this has arisen from studies of \dot{V}_E at altitude. In their beautiful paper, Rahn and Otis (18) collated data for alveolar PCO_2 and PO_2 at different altitudes for acclimatized humans and compared these with those associated with acute exposures to hypoxia (Figure 1). At 6,000 feet (1829 m) they estimated that alveolar PCO_2 fell by 4 Torr. This compared with no detectable fall at all in alveolar PCO_2 when the exposure to this level of hypoxia was acute. This study, and others like it, demonstrate that sustained hypoxia does have the power to alter the CO_2 set point. Of course, what these studies do not demonstrate is any role for the mechanism at sea level, since the fall in alveolar PO_2 was always far greater than anything that might be expected to occur through small variations in alveolar ventilation at sea level.

Studies from our laboratory on ventilatory acclimatization to hypoxia have been undertaken in a chamber in which the content of both O_2 and CO_2 could be varied up or down (13). This gave us the opportunity to expose individuals to mild hyperoxia for 8 h, and examine whether they could be de-acclimatized from the "high altitude" of sea level. Half-an-hour after this exposure had been completed, we found that end-tidal PCO_2 was significantly higher (~ 1 Torr, see Table 1) following exposure to hyperoxia than for control (20). This suggests that there is indeed some degree of "acclimatization" to the PO_2 of sea level. This study does not provide information about how large the effect might have been had the exposure been more prolonged. However, a recent study of ours (8) involving an 8-h exposure to an inspiratory PO_2 of 127 mmHg (dry gas, equivalent altitude 1894 m) gave a fall in end-tidal PCO_2 of ~ 1 Torr (see Table 1), which is around 25% of that suggested from the longer-term data compiled by Rahn and Otis.

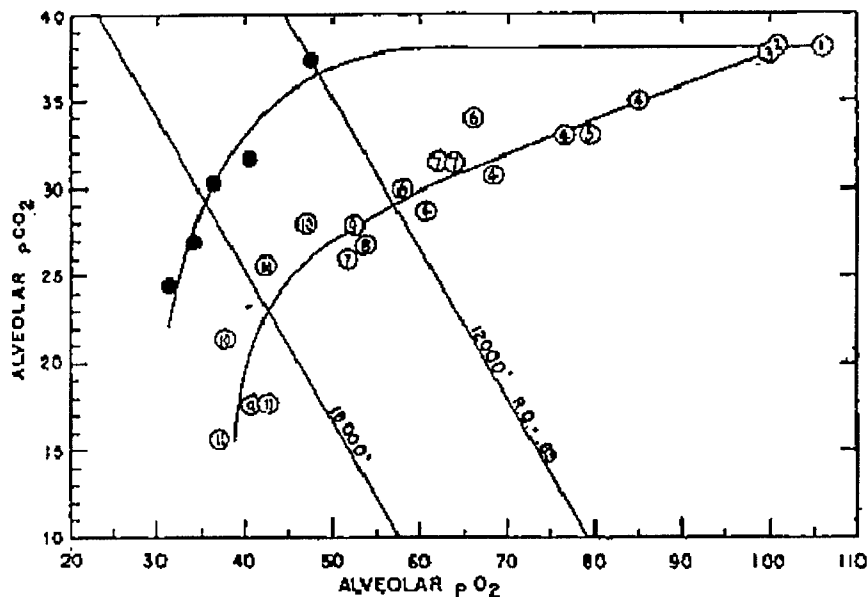


Figure 1. Alveolar PCO_2 and PO_2 for humans acutely exposed to various altitudes (solid symbols) and for humans acclimatized to various altitudes (open symbols).
From Rahn & Otis (18).

Chemoreflex sensitivities

So far, we have focussed on the ability of oxygen to vary the set point of the chemoreflexes regulating pulmonary ventilation, but an additional question is whether it can also affect the sensitivity of the chemoreflexes. Again, initial evidence has arisen from studies at altitude.

Chemoreflex sensitivity to hypoxia

Most studies (11,22,28) suggest that the acute ventilatory sensitivity to hypoxia increases with sustained exposure to high altitude. However, on their own, these studies do not indicate whether the increase results from directly from the hypoxia, or indirectly from the associated hypocapnia and respiratory alkalosis. In order to separate these influences, we compared exposures to sustained poikilocapnic hypoxia (8 or 48 h duration) with similar exposures to isocapnic hypoxia, where the respiratory alkalosis is prevented by addition of CO_2 to the inspired gas (14,24). The results from these studies indicated clearly that it was the sustained hypoxia, per se, that was responsible for the increase in acute ventilatory sensitivity to hypoxia, and that concomitant respiratory alkalosis was not required. Analogous

results have been reported in the goat where in addition it has been possible to localise the hypoxia to just a single carotid body with the rest of the animal maintained euoxic and eucapnic (1). These results provide strong evidence that sustained hypoxia at the carotid bodies increases the acute ventilatory sensitivity to hypoxia. Of further note are the observations that the nervous discharge from the carotid body progressively increases during sustained hypoxia (16), but not during sustained hypercapnia (7).

Table 1a. Effects of 8 h of mild hypoxia (inspired $PO_2 = 127$ Torr).

Condition	End-tidal PCO_2 (Torr)		Ventilation at fixed end-tidal PCO_2 (l/min)		Acute hypoxic ventilatory response (l/min/%)	
	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>
Hypoxia	39.2	38.1	11.1	14.7	0.84	1.33
Control	39.2	39.0	11.6	11.6	0.95	0.99

Data from Fatemian et al., 2001. The fall in end-tidal PCO_2 , the rise in ventilation at fixed end-tidal PCO_2 and the increase in acute hypoxic ventilatory response were all significant following hypoxia compared with the control response at $p < 0.05$. Measurements after exposure were made following 30 min of breathing room air.

Table 1b. Effects of 8 h of mild hyperoxia (end-tidal $PO_2 = 200$ Torr).

Condition	End-tidal PCO_2 (Torr)		Ventilation at fixed end-tidal PCO_2 (l/min)		Acute hypoxic ventilatory response (l/min/%)	
	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>
Hyperoxia	38.5	39.3	14.5	12.6	0.89	0.74
Control	38.4	38.4	14.0	14.3	0.83	0.80

Data from Ren et al, 2000. The rise in end-tidal PCO_2 , the fall in ventilation at fixed end-tidal PCO_2 and the fall in acute hypoxic ventilatory response were all significant following hyperoxia compared with the control response at $p < 0.05$. Measurements after exposure were made following 30 min of breathing room air.

Although these results provide evidence that the acute ventilatory response to hypoxia is enhanced following sustained hypoxic exposure, they provide no evidence that this mechanism is active through the range of values for PO_2 associated with residence at sea level. However, recently we have shown that even very mild hypoxia for 8 h (equivalent altitude 1894 m) does cause an increase in the acute ventilatory response to hypoxia (8) (Table 1). Furthermore, exposure to mild hyperoxia for 8 h reduces the acute ventilatory response to hypoxia (20) (Table 1). These results suggest that the mechanism by which sustained hypoxia modulates the sensitivity of the acute hypoxic ventilatory response does at least have the potential to act over the range of values for PO_2 associated with residence at sea level. Is there evidence that the acute ventilatory response to hypoxia actually does undergo any modulation at sea level? There are two reports demonstrating

that the between day variability in the acute hypoxic ventilatory response is substantially greater than the within day measurements of the acute hypoxic ventilatory response (21,29). Neither report considered any associated variability in metabolic acid base status to be sufficient to explain the observed variation in the ventilatory response.

Chemoreflex sensitivity to CO₂

On exposure to high altitude, it is well recognised that the ventilation-end-tidal PCO₂ relationship shifts to the left and increases in slope (3,10,15,19). As in the case of other responses to hypoxia, one question that arises is whether these changes result directly from the hypoxia, or whether they result indirectly from the associated respiratory alkalosis. Again, this issue can be approached by comparing poikilocapnic exposures to hypoxia with isocapnic exposures to hypoxia. Results from our laboratory suggest that it is the hypoxic exposure that is important rather than the associated respiratory alkalosis, at least for relatively modest levels of hypoxia up to a duration of 48 h (9,24).

Where may the sustained hypoxia be acting to increase the acute ventilatory response to CO₂? As the determinations of the acute ventilatory sensitivity to CO₂ were made under conditions of acute hyperoxia, it would seem likely that it is the central chemoreflex response to CO₂ that is affected. Furthermore, acute experiments in both animals and humans suggest that the effects of the central and peripheral chemoreflexes are independent and additive (4,25). If so, it would seem unlikely that the effects of sustained hypoxia at the carotid body underlie this effect. Despite these observations, there is some limited direct evidence from studies in the goat to suggest that the carotid bodies may be important. Engwall and Bissgard (6) have demonstrated that generalized sustained isocapnic hypoxia increases the subsequent acute ventilatory response to CO₂, whereas Weizhen et al (26) have reported that sustained hypoxia applied so that it affected the central nervous system but not the carotid body did not alter the acute ventilatory response to CO₂. Furthermore, while the evidence that the peripheral and central chemoreflexes are independent of one another in the acute setting is quite strong, this is not necessarily the case over longer periods. In particular, Pan et al (17) have demonstrated a reduction in the acute ventilatory sensitivity to CO₂ following carotid body denervation that is of slow onset, and is maximal 4 to 5 days after section of the carotid sinus nerves (Figure 2). This striking observation raises the possibility that variations in the mean level of PO₂ at the carotid body may not only influence both the set point of the respiratory chemoreflexes and the sensitivity of the peripheral chemoreflex, but that it may also modulate the sensitivity of the central chemoreflex.

CONCLUDING REMARKS

In order for the respiratory controller to function properly, it requires appropriate values for the set point and for the sensitivities of the chemoreflexes. This paper asks the question whether any self-calibrating mechanisms exist within the respiratory controller to achieve these values? It floats the hypothesis that, despite the fact that the acute chemoreflex responses at sea level are dominated by the effects of CO_2 , arterial PO_2 may nevertheless provide an important signal for long term calibration of the respiratory controller. Furthermore, we ask the question whether such a self-calibrating system is responsible for the ventilatory acclimatization that we observe in response to hypoxia. If so, it would not be a finding without precedent, for we now recognise that the polycythaemia of high altitude results from a mechanism that is active at sea level to regulate haematocrit.

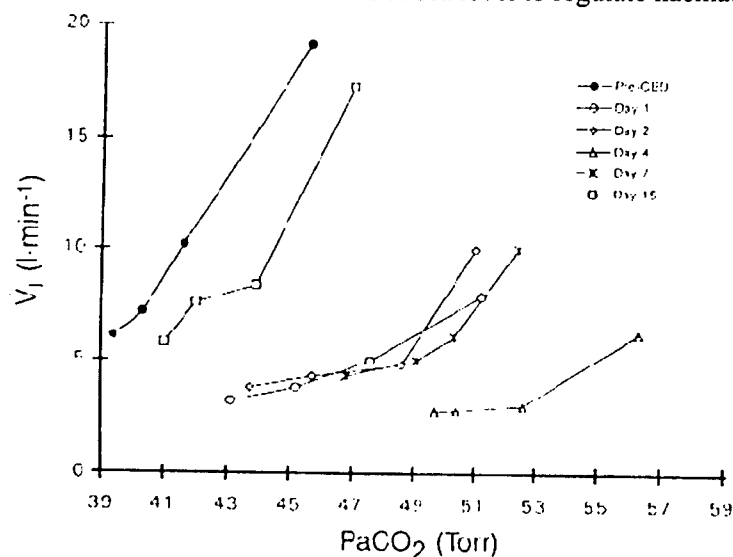


Figure 2. Relation between pulmonary ventilation and arterial PCO_2 in one goat before and for several days after carotid body denervation. From Pan et al, (17).

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Chapter 23

Roles of adenosine and nitric oxide in skeletal muscle in acute and chronic hypoxia

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Abstract: In experiments on anaesthetised rats, the roles played by adenosine and nitric oxide (NO) were determined in resting skeletal muscle in acute systemic hypoxia and during acclimation to chronic systemic hypoxia. It is concluded that adenosine acting on A₁ receptors, at least in part in an NO-dependent manner, plays essential roles in causing the dilation of proximal and terminal arterioles that helps to maintain muscle O₂ consumption when O₂ delivery is reduced by acute systemic hypoxia. It is proposed that adenosine and NO are similarly responsible for causing the tonic vasodilation that gradually wanes in the first 7 days of chronic hypoxia and that concomitantly, adenosine and hypoxia stimulate VEGF expression, so increasing venular permeability and triggering angiogenesis. By 7 days of chronic hypoxia, arteriolar remodelling is well established and within 18-21 days, substantial capillary angiogenesis alleviates tissue hypoxia. At this time, vasoconstrictor responses to the sympathetic transmitter norepinephrine are reduced, but dilator responses to adenosine released by acute hypoxia are enhanced, as may be explained by increased sensitivity to NO. Thus, preservation of tissue oxygenation is apparently associated with impaired ability to regulate arterial pressure and vulnerability to further hypoxia.

Key words: high altitude, sympathetic activity, oxygen consumption, vasodilation, endothelium, angiogenesis, vascular permeability

INTRODUCTION

It is generally accepted that systemic hypoxia induces vasodilation in resting skeletal muscle. The mechanisms underlying this response and its functional consequences have not been fully established. It has been reported

that chronic systemic hypoxia causes angiogenesis in skeletal muscle, but this has remained controversial. There is also suggestive evidence that at least during the first few days of chronic systemic hypoxia, vascular permeability in skeletal muscle is increased and there is evidence that vascular responsiveness to the sympathetic transmitter, norepinephrine, is reduced. The mechanisms underlying these changes and whether or not they are interrelated has received very little attention. The aims of the experiments described below were to investigate these issues with a view to increasing our understanding of the coordinated response evoked in skeletal muscle by systemic hypoxia.

METHODS

All experiments were performed on spontaneously breathing, anaesthetised rats, under a Project Licence approved under the Home Office (UK) Animals (Scientific Procedures) Act 1986. The methods used have been described in detail previously (6,12,23). Briefly, arterial pressure (ABP) was continuously recorded from a femoral or brachial artery and heart rate (HR) was derived from the pressure recording. Blood flow to the muscles of the hind limb was recorded from the femoral artery (femoral blood flow, FBF) by using an electromagnetic or transonic flow transducer, with non-muscular arterial branches ligated. Femoral vascular conductance (FVC) was computed on-line as FBF/ABP . In some experiments, O_2 delivery to the hind limb (DO_2) was calculated from FBF and the O_2 content measured in a sample of arterial blood, while O_2 consumption of the hind limb (VO_2) was calculated from the hind limb arterial-venous difference for O_2 content and FBF. In other experiments, the spinotrapezius muscle was prepared for intravital microscopy (23). Changes in diameters of individual arterioles and venules were measured and assessments made of changes in vascular permeability by using Evan's Blue which binds to plasma proteins and fluoresces under rhodamine illumination. By using computer-based image analysis, the ratio of interstitial: intravascular fluorescence (It:Iv) was measured by placing a rectangular window over the blood vessel and an adjacent area of skeletal muscle fibres: an increase in It:Iv indicated extravasation of plasma proteins (37).

Acute systemic hypoxia was induced by changing the inspire from 21% O_2 in N_2 to 14-6% O_2 for 3-5 minutes. Some rats were made chronically hypoxic (CH rats). They lived in a normobaric hypoxic chamber maintained at 12% O_2 for periods ranging from 1 to 28 days. At the time of the acute experiment they were removed from the chamber and breathed 12% O_2 under anaesthesia except when the inspire was changed as part of the protocol (25,34).

Pharmacological agonists and antagonists were delivered systemically, or close-arterially to the hind limb by means of a cannula placed on the ventral tail artery. In the experiments on the spinotrapezius muscle, pharmacological agents were applied topically to the muscle. At the end of some experiments, a vascular cast material was infused into the animal via the left ventricle (31). The spinotrapezius muscle was removed, dehydrated and cleared in methylsalicylate. Quantitative analysis was then performed of interbranch intervals along the arteriolar tree and of the diameters of those branches. In other experiments, leg muscles were removed, snap frozen and sectioned transversely (10). These were stained for alkaline phosphatase to demonstrate all capillaries and for succinic dehydrogenase and myosin ATPase to distinguish the three main muscle fibre types: slow oxidative, fast oxidative glycolytic, fast glycolytic. Within each muscle, the same 3-6 regions (depending on the muscle) relative to the section boundaries, were analysed by using an unbiased counting rule to assess the numbers of capillaries and size of the muscle fibres.

Mechanisms of the dilator response to acute systemic hypoxia

In the rat, acute systemic hypoxia (15-6% inspired O₂, lasting 3-5 minutes) evokes an increase FVC. The size of this response is graded with the level of hypoxia and is accompanied by a fall in ABP. This means that FBF remains more or less constant (6,12,22). These muscle vasodilator responses are greatly reduced by an adenosine receptor antagonist or adenosine deaminase indicating they are mainly mediated by adenosine (6,33). Although human subjects do not generally show a fall in arterial pressure until the level of hypoxia is severe, muscle vasodilation occurs as it does in the rat and recent evidence indicates this is partly mediated by adenosine (18).

Our direct observations on the spinotrapezius muscle have similarly shown that adenosine is responsible for the dilator responses evoked in individual arterioles and venules by systemic hypoxia. They also suggest that the terminal arterioles and the collecting venules that supply and drain the capillary bed respectively are more sensitive to adenosine than the proximal arterioles, the vessels that principally regulate muscle vascular conductance and the larger venules (Figure 1, 23). It seems highly likely that the adenosine that is released during systemic hypoxia originates from the vascular endothelium, rather than from the skeletal muscle fibres as it does during muscle contraction (see 21). The endothelial cells must experience hypoxia as a direct consequence of the fall in arterial PO₂. Endothelial cells are known to release adenosine in response to hypoxia (9,21). Moreover, if the dilation this adenosine produces is effective in improving O₂ delivery

and distribution, it would prevent hypoxia from occurring in the tissue cells (see below).

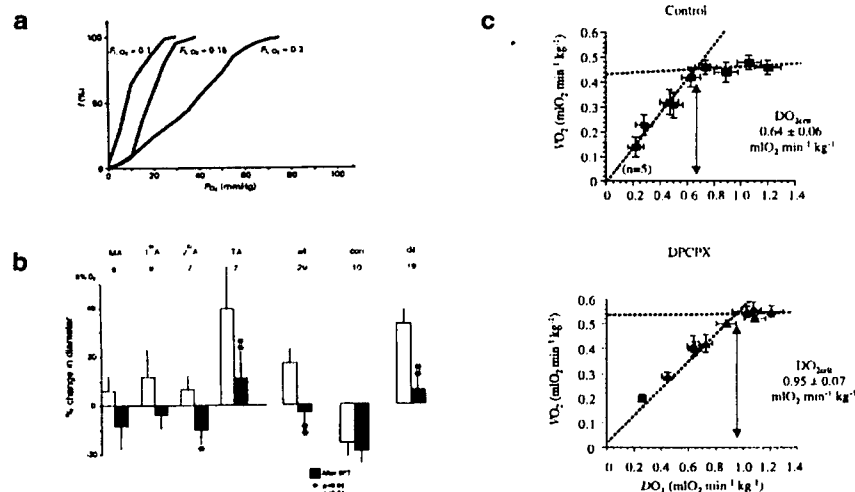


Figure 1. Effects of acute systemic hypoxia on muscle microcirculation. a) Cumulative tissue PO₂ histograms recorded in skeletal muscle of dog when inspired O₂ was reduced in stages from 30% to 15% and then to 10%. (Taken from 15). b) Responses evoked in consecutive sections of arteriolar tree of spinotrapezius muscle by acute hypoxia before and after topical application of an adenosine receptor antagonist (8-PT). MA, 1°A, 2°A, TA : main arteries, primary, secondary and terminal arterioles respectively, numbers below showing numbers of vessels and columns showing mean ± SEM for each group. All : all vessels grouped together, con, dil : those vessels that showed constrictor or dilator responses respectively to hypoxia before application of 8-PT. (Taken from 23). c) Effects of graded systemic hypoxia on DO₂ and VO₂ of rat hind limb before (above) and after (below) the adenosine A₁ receptor antagonist (DPCPX). DO_{2crit}, the DO₂ value below which VO₂ becomes dependent on DO₂ was significantly increased by DPCPX. (Taken from 13).

The availability of pharmacological antagonists specific to the adenosine receptor subtypes has meant we have been able to study their relative importance. Such experiments showed the increase in FVC evoked by acute hypoxia was attributable to stimulation of the A₁ receptors. The A_{2A} receptors apparently play no significant role even though part of the response to exogenous adenosine is mediated via A_{2A} receptors (6). The hypoxia-evoked increase in FVC was almost attenuated when nitric oxide (NO synthesis is blocked with L-NAME (L-nitro-arginine methyl ester), as was the increase in FVC evoked by exogenous adenosine and a selective A₁ receptor agonist. This indicates that the adenosine-mediated component of the response is NO-dependent (7,31).

Our most recent experiments have shown that the hypoxia-evoked increase in FVC can also be reduced by prostaglandin (PG) synthesis

inhibitors and that this is similarly true of the increase in FVC evoked by adenosine and by a selective A_1 receptor agonist (1). Following this up *in vitro*, on isolated pieces of freshly excised aorta, and using an NO-sensitive electrode to detect release of NO, we have shown that stimulation of endothelial A_1 receptors does indeed release NO in a manner that is dependent on PG synthesis and that PGI_2 can also cause NO release (28). Thus, there is good reason to suppose that *in vivo*, adenosine released from the endothelium during hypoxia acts on endothelial A_1 receptors to stimulate the synthesis of PGs which in turn activates NO synthesis, so releasing NO which dilates the arterioles. But, in other recent studies, we showed that when the baseline level of FVC was restored after L-NAME, by giving an infusion of a NO donor (inhibition of NO synthesis causes substantial vasoconstriction) then the increase in FVC evoked by systemic hypoxia was also restored (12). Moreover, this increase in FVC was greatly reduced by an A_1 receptor antagonist (13). Thus, at least when new synthesis of NO is blocked, adenosine can still produce dilation providing a background level of NO is present, presumably by acting on A_1 receptors on the vascular smooth muscle and opening ATP-sensitive K^+ (K_{ATP}) channels (8). If the muscle vasodilation that occurs during acute hypoxia is functionally important, it may not be surprising that there are a number of different, potentially overlapping mechanisms by which adenosine can exert its dilator effects.

The functional importance of the muscle vasodilation

From our direct observations on the spinotrapezius muscle it was clear that the arteriolar tree does not behave in a uniform way to systemic hypoxia: many individual proximal and terminal arterioles dilate, but others constrict (Figure 1). While the dilator responses can be mainly attributed to adenosine, the vasoconstrictor responses can be ascribed to the systemic reflex effects of hypoxic stimulation of the peripheral chemoreceptors: to an increase in sympathetic activity and to increased levels of vasopressin and angiotensin (see 19). We therefore surmised that adenosine is released in proportion to the local level of hypoxia and that the reflex vasoconstrictor effects predominate in regions where the hypoxia is most severe. Now, measurements of tissue PO_2 (tPO_2) within skeletal muscle have shown that under normoxic conditions there is considerable variability in the values recorded in different regions. However, during systemic hypoxia, the expected decrease in the mean value of tPO_2 was accompanied by marked decrease in the variability (Figure 1, 15). This raises the possibility that heterogeneity in the arteriolar responses to systemic hypoxia, especially the hypoxia-induced dilator responses has the functional consequence of making the distribution of O_2 within the muscle more homogenous. If this is the

case, then one would expect arteriolar dilation, particularly dilation of the terminal arterioles, to play an essential role in maintaining resting muscle VO_2 during systemic hypoxia and that any manoeuvre that disrupts their normal dilation would result in VO_2 becoming more dependent on DO_2 .

To test this hypothesis, we measured muscle DO_2 and VO_2 during graded levels of systemic hypoxia. In accord with findings made in larger animals, VO_2 remained constant during progressively more severe falls in DO_2 , until a critical value of DO_2 ($\text{DO}_{2\text{crit}}$) was reached. Then, VO_2 fell in linear fashion with DO_2 (Figure 1). This value of $\text{DO}_{2\text{crit}}$ was not changed by a selective $\text{A}_{2\text{A}}$ receptor antagonist, but was substantially increased and by similar extents, after administration of a selective A_1 receptor antagonist, or the NO synthesis inhibitor, L-NAME (Figure 1, 12,13). Thus, we have clear evidence that during systemic hypoxia, the dilation of terminal arterioles caused by adenosine acting on A_1 receptors to release NO, plays an essential functional role in protecting resting muscle VO_2 against the fall in DO_2 . Under normoxic conditions, the % O_2 extraction (VO_2/DO_2) by the resting muscle was <40% in our experiments and at $\text{DO}_{2\text{crit}}$ it had increased to ~80% (12), presumably because terminal arteriolar dilation effectively opened the capillary bed and decreased the diffusion distances between muscle fibres and capillaries. The fall in VO_2 that occurs below $\text{DO}_{2\text{crit}}$ must arise principally because the functional limit of the muscle fibres to extract O_2 has been reached.

Resting vascular tone in early chronic systemic hypoxia

Just as adenosine and NO are main contributors to the muscle vasodilation of hypoxia lasting a few minutes, one might expect them to be important when hypoxia is prolonged for a few days. In fact, in 1 day CH rats (1 CH rats) breathing 12% O_2 , baseline ABP was reduced and FVC increased to levels comparable with those reached in control (normoxic, N) rats breathing 12% O_2 for 3-5 minutes (12,34). Moreover, recordings made from 3 and 7 CH rats, suggested ABP and FVC gradually returned towards control levels such that in 21-28 CH rats breathing 12% O_2 , levels of ABP and FVC were very similar to those recorded in N rats breathing air (34,34). The effects of pharmacological antagonists indicated a tonic dilator influence of adenosine acting on A_1 but not on $\text{A}_{2\text{A}}$ receptors, and an increased tonic dilator influence of NO acting in hind limb muscle of 1 and 3 CH rats which had waned in 7 CH rats (35,36). However, in 21-28 CH rats breathing 12% O_2 , there was no tonic dilator influence of adenosine as is the case in N rats breathing air, and the tonic dilator influence of NO was comparable to that of N rats breath air (34,36). Thus, within 3 weeks of chronic systemic hypoxia, any tissue hypoxia within resting skeletal muscle

is largely resolved and it seems the changes that allow this to happen begin within 3 days and are already well developed at 7 days.

Vascular permeability in early chronic systemic hypoxia

It is firmly established that chronic systemic hypoxia can increase vascular permeability in brain and lungs where it can lead to cerebral and pulmonary oedema. These responses have attracted attention because they are life-threatening. However, there is also evidence that chronic hypoxia is commonly associated with peripheral oedema in limbs and face (16). In the brain, oedema has been associated with stimulation of the expression of VEGF (vascular endothelial growth factor, 41). The angiogenic action of VEGF is characterised by its effect on vascular permeability, in fact, it was first named vascular permeability factor. In experiments on rats and mice, VEGF mRNA has been shown to increase in brain and skeletal muscle within 6-8 hrs, while VEGF protein levels began to rise within 12 hrs of the onset of severe hypoxia and reached a maximum within 2-3 days (5,40). It may be noted that adenosine as well as hypoxia *per se*, is a potent stimulus for VEGF mRNA expression (14,26) and that VEGF has been shown to produce its effects on mitogenesis and vascular permeability by stimulating the synthesis of NO and platelet activating factor PAF (30,40). It was in view of this evidence that we studied the effect of chronic hypoxia on vascular permeability in skeletal muscle.

Using Evan's Blue as a means of following the extravasation of plasma proteins (see above), our results suggested there is a substantial increase in the permeability of the small venules of skeletal muscle in 1, 3 and 7 CH rats which has completely resolved in 21-28 CH rats. By contrast, there was no obvious change in permeability of the arterioles nor of the capillaries that run parallel with muscle fibres (37). This pattern of change in vascular permeability could be mimicked by topical application of human recombinant VEGF, or PAF. Moreover, the effect of exogenous VEGF was prevented by L-NAME and reduced by a PAF receptor antagonist (38). At this stage, it must be acknowledged that the increase in vascular permeability seen in CH rats may be caused by substances other than VEGF. However, it is tempting to speculate that endogenously released VEGF does increase vascular permeability in skeletal muscles in the first 7 days of chronic hypoxia and that this effect is mediated by the combined actions of NO and PAF.

Angiogenesis in chronic systemic hypoxia

Whether or not the increased vascular permeability is attributable to VEGF, the very fact that tissue hypoxia under a variety of conditions is a

potent stimulus for angiogenesis (2) was good reason to look carefully at whether angiogenesis occurs in skeletal muscle during systemic hypoxia. By quantitative analysis of vascular casts of spinotrapezius muscle, we established that in 7 CH rats there was already a decrease in the interbranch interval along proximal and terminal arterioles and in 18 CH rats, interbranch interval was even shorter (Figure 2). This suggests a progressive increase in the density of the arteriolar tree (31). Complementary results have been obtained by others who used immunohistochemistry to identify actin and myosin in blood vessel walls (28). Although muscularisation of existing capillaries was part of the explanation for the increased number of arterioles, it also seemed there was muscularisation of new capillaries, implying capillary angiogenesis may already have occurred within 7 days of chronic hypoxia (28,32). Coupled with these anatomical changes, the maximal vasodilation induced by infusion of SNP (sodium nitroprusside) an index of "structural vascular conductance" tended to be increased in 7CH rats, and in 18CH rats it was 2.5 fold greater than in age-matched N rats (32).

The question of whether or not systemic hypoxia induces capillary angiogenesis in skeletal muscle has been confused, partly because high altitude studies on animals and human subjects were often complicated by exposure to cold and/or because the individuals also took part in exercise. In addition, the results of animal studies are difficult to interpret because the CH and N animals were not properly matched for weight (growth itself changes muscle capillarity) and because the samples taken for analysis were not standardised (see 10). We tried to avoid these problems by using CH rats taken from a well-controlled environment, weight-matched to their control N rats and by analysing standardised regions within each muscle (10). The results showed that in 21CH rats there was a substantial increase in the ratio of the number of capillaries to the number of muscle fibres (capillary:muscle fibre ratio, C:F) in specific regions of both the tibialis anterior muscle which is mainly comprised glycolytic fibres, and in the soleus muscle which is highly oxidative (17 and 30% increases respectively, see Figure 2). There was no change in the size of the muscle fibres as indicated by their cross-sectional area (FCSA) in any region of either muscle, showing there was no muscle atrophy. But in both muscles, the increase in C:F occurred in the region(s) that had fibres with the largest FCSA (10, Figure 2). This novel finding strongly suggests that chronic systemic hypoxia does induce capillary angiogenesis in animals that are essentially sedentary, that the metabolic profile of the muscle fibres may influence the extent of the angiogenesis (glycolytic vs. oxidative), but that the strongest stimulus is muscle fibre size (FCSA). It is likely that capillary angiogenesis occurs in those regions of muscle where the diffusional distance for O₂ from capillary to muscle fibre is greatest and therefore in which chronic systemic hypoxia

causes the lowest levels of tPO_2 : our mathematical analysis indicated the critical tPO_2 for triggering angiogenesis may be between 10 and 15 mmHg (10). Thus, these findings suggest that the regions of muscle that have the lowest level of tPO_2 may not only be regions where arteriolar and venular dilation is most profound during acute systemic hypoxia (see above), but also the regions where angiogenesis is triggered when hypoxia is prolonged.

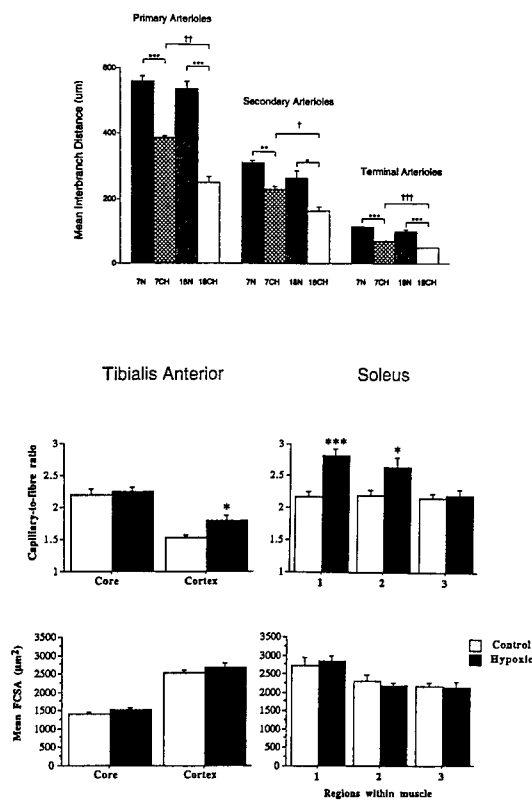


Figure 2. Effects of chronic systemic hypoxia on arteriolar branching and capillarity in skeletal muscle. Above : Mean interbranch interval along primary, secondary and terminal arterioles in 7N, 7CH, 18N and 18CH rats. Each column shows mean \pm SEM. (Taken from 32). Below : Capillary : fibre ratios and mean cross-sectional area of fibres (FCSA) in core and cortex of tibialis anterior and in 3 defined regions of soleus; each column shows mean \pm SEM. (Taken from 10).

Vascular responsiveness in chronic systemic hypoxia

The discussion above indicates that within 3-4 weeks of chronic systemic hypoxia, skeletal muscle and its vasculature have, in some respects, normalised. An increase in the size of the arteriolar tree and the capillary bed together with an increase in haematocrit apparently ameliorate the fall in tPO_2 so that under resting conditions when breathing 12% O_2 , there is no tissue hypoxia, at least as evidenced by the lack of effect of adenosine receptor antagonists on baseline FVC or arteriolar diameter (25,34). However, the very fact that the baseline FVC of 21-28CH rats breathing 12% O_2 is comparable to that of N rats breathing air, even though the CH rats have a larger structural vascular conductance (see above) suggests that the basal tone of individual arterioles must be greater in the CH rats (32,34). Further, when 21-28CH rats were exposed to a further hypoxic challenge by switching the inspire from 12 to 8% O_2 , they showed increases in FVC that were almost as large, and increases in arteriolar and venular diameter that were as large, as occurred in N rats when they were switched from air to 8% O_2 . Moreover, as in N rats, these dilator responses were largely mediated by adenosine (23,34, Figure 3). Clearly, the situation is complicated, but these results suggest that the dilator influences of acute hypoxia, of adenosine in particular, may be greater in CH rats and/or the constrictor influences of acute hypoxia are weaker.

Some evidence that the latter may be true comes from experiments on chronically hypoxic patients: they showed depressed muscle vasoconstrictor responses to infused noradrenaline and depressed reflex vasoconstrictor responses to lower body negative pressure such that they were not able to maintain ABPC (17). Similarly, 21-28CH rats showed smaller increases in TPR in response to graded concentrations of the vasoconstrictors the α_1 receptor agonist phenylephrine, vasopressin and angiotensin than N rats while *in vitro*, their aortae showed depressed contractile responses to these substances (11).

In an attempt to elucidate these phenomena, we have used intravital microscopy to construct dose-response curves to topical application of the sympathetic neurotransmitter norepinephrine (NE) on individual arterioles. In the spinotrapezius muscle, arteriolar dilator responses to low doses of NE, which are mediated by β adrenoreceptors (19) were abolished in CH rats, while the constrictor responses to NE were greatly depressed: NE_{max} was reduced with no effect on the dose that evoked 50% of NE_{max} (Figure 3). Similar results were obtained in arterioles of the intestinal mesentery (24, Figure 3). Since these arterioles have virtually no parenchymal cells around them, the depressed response to NE must reflect a change in the responsiveness of the arteriole, rather than the action of a substance released from the tissue cells.

Knowing the strong counteracting influence NO can have on vasoconstrictor responses, an obvious possibility was that NO was involved. Topical application of L-NAME to the mesentery of N rats had little effect on baseline diameter or NE_{max} as previously described in intestinal microcirculation (27). By contrast in 21-28CH rats L-NAME caused a small reduction in baseline arteriolar diameter and greatly accentuated NE_{max} so that it equalled that evoked in the N rats (Figure 3). We obtained essentially similar results when we measured isometric tension in rings of iliac arteries, arteries that supply hind limb muscle. Iliac arteries from 21-28CH rats showed greatly reduced NE_{max} values relative to N rats, whether the comparisons were made at PO_2 values of 100 mmHg or 55 mmHg (approximately the PaO_2 pertaining in the N and CH rats respectively). Further, L-NAME had no significant effect on baseline tension, but potentiated the contractile responses in the arteries from the CH rats so that the NE_{max} values recorded in arteries from CH and N rats were similar. Importantly, removal of the endothelium from the arteries of the CH rats also normalised the NE_{max} relative to N rats and under these conditions L-NAME had no further effect (3,4).

Taken together these results suggest that NO released from the endothelium exerts a much stronger counteracting influence on NE-evoked vasoconstriction in 21-28CH rats than N rats. Given that L-NAME had no greater effect on baseline FVC or ABP in 21-28CH than N rats (see above), it would seem that the tonic synthesis of NO is *not* generally increased in CH rats, or that if it is, its dilator effect is counteracted by some other factor. However, the evidence is consistent with the effect of stimulated release of NO being increased in 21-28CH rats. This might occur if there is an increase in expression or activity of NOS in the endothelial cells, or an increase in the sensitivity of the vascular smooth muscle to NO. Whatever the mechanism, an augmented dilator influence of NO may contribute to the depressed vasoconstrictor responses to exogenous NE and to sympathetic activity if nerve-released NE has access to adrenoreceptors on the endothelium. It may also contribute to the augmented dilator responses to acute systemic hypoxia, for adenosine released by acute hypoxia may cause greater NO-induced dilation. These proposals clearly leave open the possibility that the effects of other vasoconstrictor and dilator influences upon skeletal muscle vasculature are also changed by chronic systemic hypoxia in ways that are not dependent upon NO. Interestingly, syncope is common in healthy individuals who are acclimatising to chronic systemic hypoxia (20,39).

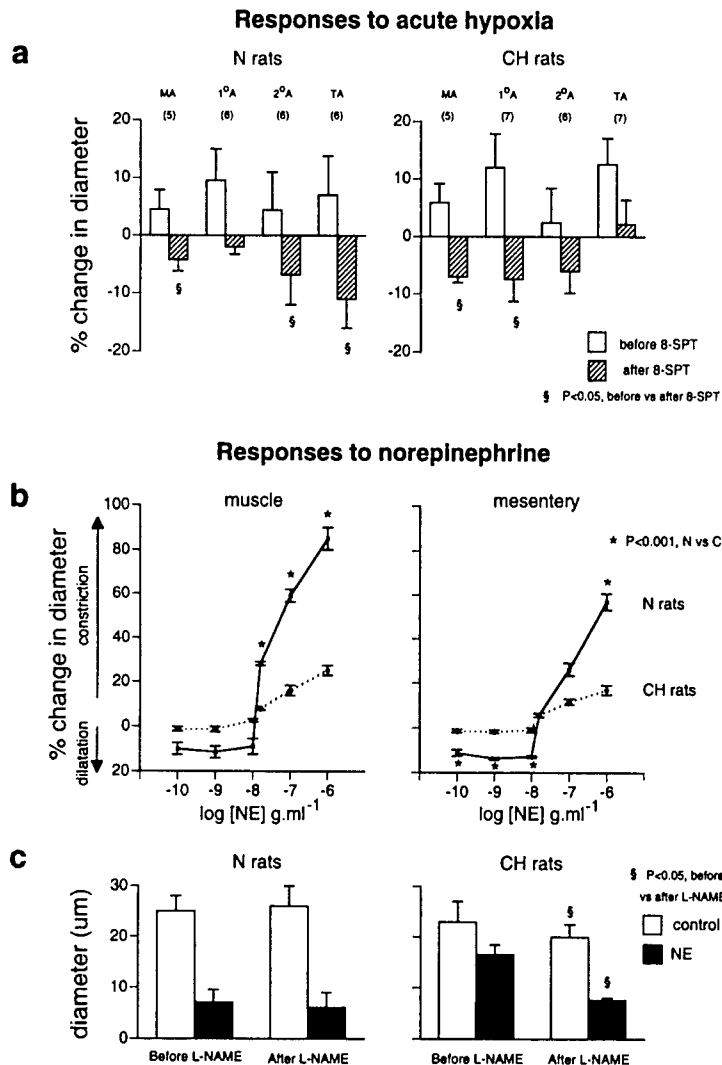


Figure 3. Effects of chronic systemic hypoxia on responses to acute hypoxia and to norepinephrine (NE). a) Responses evoked in consecutive sections of the arteriolar tree of N and CH rats by acute hypoxia before and after the adenosine receptor antagonist 8-SPT. Abbreviations as in Fig 1. (Modified from 25). b) Dose response curves to NE in arterioles of spinotrapezius muscle and intestinal mesentery in N and CH rats. Control diameters of muscle arterioles were $16 \pm 0.5 \mu\text{m}$ and $16 \pm 0.6 \mu\text{m}$ in N and CH rats respectively, while control diameters of mesenteric arterioles were 23 ± 1.5 and $22 \pm 1.4 \mu\text{m}$, respectively. c) Diameter changes evoked by NE in mesenteric arterioles of N and CH rats before and after topical application of L-NAME. Open and black columns show control diameter and diameter at NE_{max} (respectively), mean \pm SEM. (Data from 20, 24).

CONCLUSIONS

The results discussed above suggest that adenosine plays a major role within skeletal muscle both in acute and chronic systemic hypoxia. In acute hypoxia, adenosine, acting on A₁ receptors at least in part by releasing NO from the endothelium, is responsible for the coordinated dilation of the proximal arterioles that increases FVC and maintains gross muscle DO₂, and for the dilation of the terminal arterioles that redistributes the available O₂ through the capillary network and maintains VO₂ until O₂ diffusion distances from capillaries become limiting. In the first few days of chronic systemic hypoxia, the continuing release of adenosine and NO maintain a tonic dilator influence on skeletal muscle that maintains DO₂ while other adaptive changes develop. During this time, we propose that hypoxia- and adenosine-stimulated expression of VEGF increases venular permeability in a NO- and PAF-dependent manner and stimulates angiogenesis. The consequent development of new capillaries and the muscularisation of capillaries to form arterioles, together with the increasing haematocrit, mean that capillary diffusion distances are reduced and tissue oxygenation is preserved within 1-3 weeks of chronic hypoxia, such that tonic release of adenosine is abolished. But, despite, or may be because of, these structural and haematological changes, the vasoconstrictor influence of NE is depressed in established chronic hypoxia and the vasodilator influence of the adenosine released by further acute hypoxia is enhanced, in ways that may be attributed to an increased influence of NO released from the endothelium. This may be associated with an impaired reflex control of arterial pressure by the baroreceptor reflex and vulnerability to the dilator effects of further hypoxic challenges.

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Chapter 24

The pVHL-HIF-1 system

A key mediator of oxygen homeostasis

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Abstract: Matching oxygen consumption and supply represents a fundamental challenge to multicellular organisms. HIF-1 is a transcription complex which is emerging as a key mediator of oxygen homeostasis. HIF-1 controls the expression of many genes, including erythropoietin, angiogenic growth factors, glucose transporters and glycolytic enzymes. The HIF-1 complex, which contains an α and β subunit (both basic helix-loop-helix proteins of the PAS family) is formed in hypoxia and modulates gene expression through hypoxia response elements. Regulation involves ubiquitin-mediated oxygen-dependent destruction of the alpha subunit. Oxygen-regulated destruction of HIF- α requires the von Hippel Lindau tumour suppressor protein (pVHL). pVHL acts as the recognition component of a ubiquitin E3 ligase complex which binds HIF- α . Loss of pVHL function, which results in constitutive activation of the hypoxic response, is important in the development of clear cell renal cancer, where both copies of the gene are usually inactivated. The importance of the VHL-HIF system in multicellular organisms is supported by conservation in the nematode *C. elegans*. Understanding the events resulting in HIF activation should provide novel therapeutic targets. This would be useful in preventing angiogenesis in cancers and promoting adaptive changes in hypoxic / ischaemic tissue.

Key words: hypoxia-inducible factor-1, von Hippel Lindau, transcriptional regulation, angiogenesis

INTRODUCTION

Oxygen is a fundamental requirement for cellular respiration, and balancing oxygen delivery and requirements throughout the organism

underlies a large number of homeostatic responses. Recently it has become clear that many of these involve regulation of gene transcription, mediated by a complex termed HIF-1 (38). HIF-1 was originally identified as a transcription complex binding to an oxygen-responsive enhancer element located 3' to the erythropoietin gene (49). The HIF complex was purified, and shown to contain an alpha and beta subunit, both of which are basic-helix-loop-helix transcription factors, which were members of the PAS domain family (47). HIF-1 β had already been recognised as the aryl hydrocarbon receptor nuclear translocator (ARNT), and is a constitutive nuclear protein. It is now appreciated that both the alpha and beta subunits are members of multiprotein families (HIF-1 α , HIF-2 α , HIF-3 α and ARNT, ARNT-2 and ARNT-3).

The oxygen-responsive subunit of the HIF-1 complex is HIF1- α or HIF-2 α , which are primarily regulated by oxygen-dependent destruction mediated by the ubiquitin-proteasome pathway (11, 37, 50). A recent development is that this has been shown by our laboratory to be mediated by specific recognition of HIF- α subunits by the VHL tumour suppressor protein (27).

Erythropoietin regulation and recognition of a widespread oxygen-sensing mechanism

The erythropoietin response is a striking example of a homeostatic response to oxygen (16). In response to anaemia, or hypoxia such as that encountered at altitude, production of this circulating hormone is increased, leading to increased red blood cell production and increased oxygen-carrying capacity. The principal cells producing the hormone after birth are the fibroblasts of the renal cortex and outer medulla (24). Interestingly, perhaps because of the specificity of action of erythropoietin itself, the initial prejudice was that the underlying oxygen-response system would be private to the erythropoietin response. However, the alternative hypothesis, that the underlying response system would act in other cells and influence other responses has proved correct. This was first established in experiments which showed that the oxygen-responsive enhancer from the erythropoietin gene was hypoxia responsive in all cell types studied (26). Since these cells did not generally express the erythropoietin gene, this strongly suggested that the underlying oxygen-response system was modulating expression of other genes. Subsequently HIF-1 was identified, and is regulated by oxygen in all cells studied, with the notable exception of those lacking the VHL tumour suppressor gene (see below).

HIF-1 responsive genes

As predicted from these findings, HIF-1 has been found to regulate the expression of genes other than erythropoietin. Examples include genes involved in glucose metabolism, angiogenesis, vascular tone and decisions concerning cell proliferation/apoptosis (38). The role of HIF in regulation of a particular candidate gene can be examined readily, based on certain characteristics of the HIF response system, and genetic resources with specific defects affecting the HIF pathway. In cultured cells, HIF is activated progressively as oxygen concentration is reduced, reaching maximal activation at ~ 0.5% oxygen. HIF activation occurs rapidly, within minutes of exposure to hypoxia. Accumulation of target gene mRNA and protein product typically continues for many hours, effectively integrating the HIF response. In addition to responding to hypoxia, HIF is also activated by exposure of cells to iron chelators or certain transition metals, eg cobaltous ions. Thus these stimuli also induce expression of HIF target genes (8, 48). The HIF recognition sequence has been characterised and a consensus formulated as 5'-RCGTG-3'. Flanking sequence can be searched for matches to the consensus, and candidate sites can then be tested for oxygen-responsiveness and HIF-dependent function in gene transfer experiments, and for HIF binding in electrophoretic mobility shift assays. Several cell culture sublines with defects in the HIF system are now available, and offer a powerful method of ascribing responses to the HIF system.

(1) Murine hepatoma (Hepalclc7) sublines selected for defects in the xenobiotic response. One complementation group of these lacks ARNT/HIF-1 β , and thus is defective in the HIF response (10, 52).

(2) A Chinese hamster ovary subline, Ka13, which was selected from a randomly mutagenised pool of cells on the basis of defective response of an HRE-driven transgene and has a defect in HIF-1 α (53). Neither the parental line (CHO-K1) or Ka13 express HIF-2 α .

(3) Murine cells (MEF and/or ES cells) with targeted inactivation of HIF-1 α , ARNT/HIF-1 β or VHL (3, 7, 14, 23, 31, 35, 36, 45). Although the effects on mouse development of inactivating HIF-2 α have been determined (see below), cell lines with inactivation of both HIF-2 α alleles have not yet been reported.

(4) Renal carcinoma cell lines with defective VHL function. These have constitutive activation of the HIF system with little or no further response to hypoxia. Sublines stably re-expressing pVHL show normal regulation of HIF (27).

To date, identification of HIF target genes has largely been on the basis of testing candidates identified because of a plausible reason for oxygen-responsiveness. The increasing facility of unprejudiced techniques for

identifying differentially expressed transcripts and proteins is changing this approach, and a number of HIF target genes have now been identified by our group using these methods (29, 55). Overall, it is clear that a large number of genes are regulated by HIF, and that the actual response in terms of the genes influenced, the amplitude, and even the direction of the response shows major variability according to cell background.

Role of HIF-1 in mammalian physiology and pathophysiology

Determining the role of HIF in physiological and pathological processes is a rather different challenge. Important tools for investigating this include sensitive, specific detection of HIF activation, and the ability to manipulate the system precisely – either through genetic means or with specific substances. Detection of HIF activation can be achieved with immunolabelling of HIF- α subunits (43, 58), since the presence of HIF- α protein correlates directly with HIF activation. In addition, detection of the products of suitable target genes can be used for this purpose; for example, our laboratory has recently shown that the surface membrane carbonic anhydrase CA9 is an excellent marker of HIF activation in a range of tumours (54). Our knowledge of the role of HIF is evolving rapidly. Progress in a few key areas is summarised below.

(1) Development

This has been addressed in mouse knockout experiments, which establish that homozygous inactivation of HIF-1 α , HIF-2 α , HIF-1 β /ARNT, or pVHL all result in embryonic lethality (7, 14, 23, 45). This establishes that the HIF system is essential for embryonic development. Furthermore, it demonstrates the two α subunits are not redundant.

(2) Pulmonary hypertension

Mice heterozygous for HIF-1 α are resistant to development of pulmonary hypertension in response to hypoxia compared to wild type mice, establishing that the HIF pathway can influence this process and suggesting that genetic variation in HIF responses could be important in disease susceptibility (56).

(3) Tissue ischaemia

Immunodetection of HIF- α subunits has shown that HIF is activated in myocardial ischaemia (21). In addition, in an experimental study myocardial

revascularisation was promoted by a polypeptide (PR-39) which was shown to stabilise HIF- α (22).

(4) Tumour biology

Particular interest has focussed on the role of HIF in tumour biology. Three important observations contributed to this. First, regions of low oxygen tension are very frequent in malignant tumours, reflecting the fact that tumours easily outstrip their vascular supply, and also that the interstitial pressure in tumours is increased and the vasculature is easily compressed. Second, genes found to be HIF responsive showed striking similarity to those associated with malignant tumours – notably those involved in glycolysis and angiogenesis. Third, VEGF was found to be expressed in the hypoxic regions of particular malignant tumours (40). Mindful of this, we examined whether HIF might have an important role in tumour biology by comparing tumour xenografts of murine hepatoma cells with and without HIF-1 (24). That study established that HIF had major effects on expression of genes involved in glucose metabolism and angiogenesis, and also that it could have effects on tumour growth. Since HIF had a positive effect on tumour growth we considered that in certain tumours HIF might be constitutively activated. One candidate was the VHL tumour suppressor gene, which had been shown to affect the hypoxia-inducible genes VEGF and GLUT-1 (12). Cells lacking *pVHL* had been found to have a high level of expression of these genes which was not enhanced in hypoxia (in contrast to all other cell lines). Furthermore, re-expression of *pVHL* in these cells led to suppression of the normoxic level of GLUT-1 and VEGF (12). We found that clear cell renal carcinoma cells with defective *pVHL* function had constitutive activation of the HIF pathway (27). On the one hand, this has provided an important step towards understanding the molecular events underlying oxygen-sensitivity of the HIF system (see below). It has also provided a compelling link between the HIF pathway and the malignant phenotype, since *pVHL* loss results in direct activation of HIF and carries a very strong disposition to malignancy (the lifetime risk of renal carcinoma in individuals with a germline mutation in the VHL gene is over 70%) (17). This suggests that constitutive activation of HIF may constitute a gate-opener to the development of malignancy in renal epithelial cells.

Another important line of investigation implicating HIF in malignant tumours pursued by ourselves and others has been the immunodetection of HIF- α subunits. While HIF- α subunits are only expressed at a low level, or are undetectable in normal tissues, they are present at a high level in over 50% of malignant tumours (43, 58). Furthermore, HIF activity appears to increase with malignant progression (6, 57). HIF is a powerful activator of angiogenesis, and development of sustained angiogenesis is an important

aspect of the malignant phenotype (9). An interesting paradox is that it is difficult to see how enhanced angiogenic capacity could be selected for in an evolving tumour, since it could be argued that any cell transmitting angiogenic signals might benefit its neighbours as much as itself. We suggest that the angiogenic phenotype may, in fact, be co-selected when another property which does enhance single cell-survival is selected for. The HIF system would provide an example of a pathway which could result in co-selection of the angiogenic phenotype. In this model, a downstream property (such as glycolytic capacity) would confer enhanced survival, which would result in selection of cells with increased HIF activation thus co-selecting for enhanced angiogenic signalling.

Although there could be a *prima facie* case for regarding HIF- α as an oncogene, we suspect this may not be appropriate since no activating mutation of a HIF molecule per se has yet been described in cancer. In fact it is striking that in a very wide range of cancer cells, the HIF pathway remains sensitive to oxygen. Since it is clear that HIF activation offers potential advantages to cells, this argues strongly that balancing pressures select against constitutive HIF activation in tumours. Several other lines of evidence provide some support for this view including the following. First, HIF target genes with antiproliferative and pro-apoptotic actions have been identified (2, 55). Second, hypoxia-induced apoptosis has been shown to be HIF-1 α dependent in embryonic stem cells (3). Third, in one study xenografts of ES cells lacking HIF-1 α grew more rapidly than wild type cells (3).

If this is the case, what leads to activation of the HIF system in over half of all malignant tumours? In our view, a likely explanation is that HIF activation in these circumstances is a predictable consequence of features which are being selected for in the evolution of the malignant tumour. One route resulting in this would be that cells selected on the basis of rapid proliferation due to loss of tumour suppressor genes and/or activation of oncogenes will outstrip the available oxygen supply with consequent hypoxia and HIF activation. A second link is likely to be that enhanced proliferation itself leads to amplification of the HIF response. Teleologically this is attractive, since proliferation carries a predictable energy cost. There is considerable evidence supporting such a link between proliferation and an increased HIF response. Thus activation of several oncogenes (Ha-ras, Myc and Src), loss of tumour suppressor functions (p53, PTEN) and exposure to different growth factors (insulin, IGF-1 and -2, angiotensin II) have been reported in a range of studies to increase HIF activity in normoxia and hypoxia, with a preserved hypoxic response (for review see 39; for a recent relevant study see 34).

These are examples of mammalian developmental and disease processes in which HIF has already been directly implicated. Given the central importance of oxygen in cellular metabolism it is probable that there will be many others.

An evolutionary approach to HIF

An important issue is the extent to which the HIF response is conserved in evolution. Previously we reported that a HIF binding complex was present in hypoxic cells from *D. melanogaster* (28). Furthermore expression of the gene encoding the glycolytic enzyme PGK was regulated by oxygen in these cells. Subsequently we showed that the abundance and transactivating activity of *Drosophila* SimA protein (which is homologous to HIF- α subunits) is increased in hypoxia (1). *D. melanogaster* does not have a circulation, and distribution of oxygen is distributed by a network of air tubes, and another member of the bHLH-PAS transcription factor family, *Trachealess*, is necessary for proper formation of this network (13, 51).

Recently we have performed studies in the nematode *C. elegans*. The complete genome of this organism has been sequenced and we first identified the gene with most similarity to HIF- α subunits. We have raised antibodies to the protein product, and found that this is virtually absent in worms under standard laboratory conditions. In contrast, exposure of worms to hypoxia or a penetrant iron chelator leads to a dramatic increase in the amount of this protein. We next examined the role of the VHL orthologue in HIF regulation in the worm, by studying a mutant worm selected for a mutation in the VHL gene. Exactly as we found in human carcinoma cells, loss of the VHL gene results in constitutive stabilisation of HIF α subunits. Interestingly, and in contrast to the effects in the mouse, VHL defective worms are viable and do not have a striking phenotype under standard culture conditions.

Conservation of the system in primitive multicellular organisms provides another line of evidence for central importance in responses to oxygen. As in mammalian cells there is a rather surprising lack of redundancy. Whereas in mammals the system has a key function in determining internal organisation (being required for development, remodelling and dynamic regulation of the vasculature) in the nematode it appears not to be important in internal organisation. This may relate to the much wider range of external oxygen concentrations faced by the nematode.

Identification of the system in *C. elegans*, which allows forward and reverse genetic approaches with relative ease, allows a genetic approach to understanding the molecular events regulating HIF- α stability in response to changes in oxygen tension.

HIF-1 regulation

Considerable progress has been made in understanding how HIF- α is regulated by changes in oxygen. Although nuclear translocation and transactivator recruitment are also regulated, the dominant mechanism is rapid destruction of HIF- α subunits in the presence of iron and oxygen. This is mediated by a transferrable internal oxygen-dependent degradation domain. We established that a region of 34 amino acids (residues 549-582) from HIF-1 α is sufficient to convey this oxygen regulated destabilisation (33).

As mentioned above, we found that the HIF pathway is constitutively active in renal carcinoma cells lacking pVHL. We showed that this is due to stabilisation of HIF- α subunits (27). Furthermore, we found that pVHL and HIF- α are physically associated in the presence of iron, oxygen and inhibitors of the proteasomal pathway (27). pVHL forms part of a multiprotein complex with elongin C, elongin B, rbx1 and cul-2 which shows extensive sequence and structural similarities to a family of ubiquitin E3 ligase complexes (18,32,41,42). Bringing these two lines of investigation together, it has recently been shown by ourselves and others that the β domain of pVHL interacts directly with the oxygen-dependent degradation domain of HIF- α subunits and acts as the recognition component of a ubiquitin ligase complex (5,19,30,44). Strikingly, a wide range of missense VHL mutations that are associated with familial predisposition to haemangioblastoma or sporadic renal carcinoma all interfere with pVHL's ability to regulate HIF- α . In contrast, rare missense germline VHL mutations that predispose to pheochromocytoma without other phenotypic consequences do not alter the ability to capture or ubiquitylate HIF- α (4). This supports the concept that HIF activation is causally linked to development of haemangioblastoma and clear cell renal carcinoma. However, pVHL has been reported to have other functions, which may well be involved (22, for review see 17).

Recognition of HIF- α by pVHL is prevented by iron chelators, providing insight into how iron chelation activates the HIF system. This also suggested to us that mechanisms influencing recognition of HIF by pVHL could be central to understanding how oxygen sensitivity is achieved. We have recently developed in vitro assays in which recombinant proteins containing HIF- α sequence are tested for recognition by pVHL. These experiments have shown that recognition of HIF- α is conditional on a post-translational modification, which requires cell extract, is enzymatic and is dependent on iron and oxygen (15).

Possibilities for therapeutic intervention

Potentially, manipulating the HIF system could be beneficial in a wide range of disease processes. There are already examples of animal models in which potentiating the HIF response improves outcome in ischaemia (22, 46). Furthermore, interfering with the HIF system may be helpful in cancer, and xenograft experiments suggest that this is achievable and can reduce growth of model solid tumours (20).

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Chapter 25

Interval hypoxic training

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Abstract: Interval hypoxic training (IHT) is a technique developed in the former Soviet Union, that consists of repeated exposures to 5-7 minutes of steady or progressive hypoxia, interrupted by equal periods of recovery. It has been proposed for training in sports, to acclimatize to high altitude, and to treat a variety of clinical conditions, spanning from coronary heart disease to Cesarean delivery. Some of these results may originate by the different effects of continuous vs. intermittent hypoxia (IH), which can be obtained by manipulating the repetition rate, the duration and the intensity of the hypoxic stimulus. The present article will attempt to examine some of the effects of IH, and, whenever possible, compare them to those of typical IHT. IH can modify oxygen transport and energy utilization, alter respiratory and blood pressure control mechanisms, induce permanent modifications in the cardiovascular system. IHT increases the hypoxic ventilatory response, increase red blood cell count and increase aerobic capacity. Some of these effects might be potentially beneficial in specific physiologic or pathologic conditions. At this stage, this technique appears interesting for its possible applications, but still largely to be explored for its mechanisms, potentials and limitations.

Key words: intermittent hypoxia, altitude, blood pressure, heart rate variability, chemoreceptors, ventilation, preconditioning

INTRODUCTION

Interval hypoxic training (IHT), a technique developed in the former Soviet Union, consists of repeated 5-7min of steady (in the range of 9-12%) or progressive hypoxia (down to 5-7%), interrupted by equal periods of recovery. It was used to increase the resistance to hypoxia to radiations, to train competitive athletes, to adapt to high altitude, and to treat many different disorders. Only recently a few western groups started evaluating

the effects of these techniques. The first data available refer to effects of IHT on exercise capacity, red blood cell count, ventilatory control, and the autonomic nervous system.

IHT is a specific condition of intermittent hypoxia (IH). Differences relating continuous hypoxia to IH, in general, and more specifically to IHT, are still poorly understood. These differences depend on many different aspects, such as total duration of hypoxia, and the number, duration and intensity of hypoxic exposures. Growing evidence suggests that the effects elicited by manipulating these elements may be quantitatively and even qualitatively different. The present article reviews the Russian experience, examines some of the specific effects of intermittent vs. continuous hypoxia, and, whenever possible, compares them to the known effects of IHT.

THE RUSSIAN EXPERIENCE

Studies on IHT appeared in the Russian literature since about a decade ago. The approach of different groups, with little exceptions, seems to be quite standardized, suggesting that the basic concepts and experiments were probably elaborated by experimental studies done much earlier; unfortunately little of this essential work appears traceable. Most of the works have been published only in the Russian literature, and many in Russian, so they are difficult to access by western readers. The Hypoxia Medical Journal, edited by the Moscow Hypoxia Medical Academy, is published in English and reports most of the experiences of the Hypoxia Medical Academy and other Russian groups. This makes part of the IHT material more accessible.

Most of the effects of IHT, as reported in the Russian literature, relate to an increase in oxygen transport and utilization. The former include increase in erythropoietin (Epo) and red blood cell (RBC) production (32), increase in ventilation and sensitivity to hypoxia; the latter involves biochemical changes at the cellular and molecular levels, with increased oxidative capacity of muscle mitochondria. Good results have been claimed in treatment of asthmatic children (72). These may arise from the observation of the reduced ventilatory drive often reported in asthma (76) and of its adverse prognostic significance when associated to reduced perception of dyspnea (47). The IHT has been used to train competitive athletes (68) and to adapt to high altitude (28). The effectiveness of training in either steady or intermittent hypoxia is still controversial, but the advantages of spending some time in an hypoxic environment are now rather established, at least in terms of increased oxygen transport and anaerobic capacity (13, 53). Effects of IHT on angiogenesis have been reported in the human myometrium of pregnant women (69). At least in the striate muscle, this concept has been

established and confirmed in the western literature (40). Some of these effects may be due to adaptation to stress (42); these involve an IHT-induced decrease in the rise of epinephrine and glucose in pregnant women before and immediately after Cesarean delivery (1, 2, 65). A preconditioning (25) and anti arrhythmic effect could be observed in rat heart (5), and a reduction of anginal attacks has been reported in cardiopathic subjects (18). Another line of research reports increased antioxidant response of the organisms to free radical generation by IHT (60, 2,65), and increased resistance of the heart to ischemic and reperfusion arrhythmias (59). Potential applications are thus of great interest.

Many of the concepts reported in those articles are not exclusive of the Russian experience, but could be found by careful analysis of the western literature on IH. However, in western laboratories research on continuous or intermittent hypoxia started from different perspectives, thus making direct comparisons difficult. A good example of this is the relationship between hypoxia and blood pressure (see below). Whenever a comparison is possible, the effects reported in the Russian literature are in general in agreement with the experimental evidence accumulated so far on IH.

THE WESTERN EXPERIENCE: EFFECTS OF IH ON THE OXYGEN TRANSPORT SYSTEM AND ENERGY SUBSTRATE UTILIZATION

In the western literature IH has been widely studied, with emphasis on specific effects of IH, on the oxygen transport, and on the cardiovascular system, mainly in an attempt to explain the role of spontaneously occurring IH (obstructive sleep apnea) in the genesis of stable hypertension.

Ventilatory response and IH

One of the main factors believed to describe the possibility for living organisms to adapt to hypoxia is the increase in ventilation mediated by the peripheral chemoreceptors (hypoxic ventilatory response, HVR). The role of the HVR in sports, short-term adaptation to high altitude or to chronic hypoxia is controversial, nevertheless this reflex may play an important role in specific situations and in clinical medicine. It is likely that some of the effects of IHT could be due to an increased HVR. Clinical conditions such as asthma in children (47,76), or specific autonomic diseases (such as familial dysautonomia, 21) may benefit from an increase in HVR; similarly, an increased HVR may improve working capacity during short-term exposure to high altitude.

Our experience on the effects of IHT on HVR

Several studies evaluated whether exposure to IH can increase HVR (6, 46,52), but the hypoxic stimulus was associated to physical training, so it is not known whether a similar effect can be obtained by IH alone. We therefore assessed whether a 2 weeks of IHT could modify the sensitivity of the chemoreflex to hypoxia in a group of healthy subjects (7). The study was carried out at the Bogomoletz Institute of Physiology of Kiev, in 18 healthy male subjects randomly assigned either to training (12 subjects, 4-5 rebreathing session of 5-7 min each, PAO_2 down to 40-35mmHg, separated by equal periods of recovery, in 1h, for 2 weeks) or control (6 subjects, same sessions but breathing air) groups. Resting respiratory parameters were similar in the control and IHT groups and remained unchanged after training. After training, minute ventilation was markedly increased in the training group at the end of rebreathing, as compared to before training and to the control group at the end of rebreathing. Sham training did not modify minute ventilation during hypoxia. HVR increased in the training group compared to the pre-training values and to the control group (in which we observed a non-significant tendency to increase). Thus a period of 2 weeks of IHT is capable of increasing the HVR in a group of healthy subjects. Although no direct comparison is possible with any of the data published in the western literature (where IHT alone has never been evaluated before for this purpose), these findings are consistent with a definite increase (6,52) or a tendency toward an increase (46) in HVR previously observed in subjects undergoing physical training in hypobaric hypoxia for 30-120 min/day. In those subjects, however, the hypoxic period was limited to 1 time per day, and the increase in HVR might have been counteracted by the opposite effect exerted by physical training (46,52).

Causes of the increase in HVR during IH

The reasons for this increase in HVR are difficult to explain; possible causes include a generic, non specific effect of "training", by which the subjects learn to react more and more intensively to the same stimulus (42). Also, continuous practice of intensive respiration can lead to training of the respiratory muscles, hence resulting in easier practice of hyperventilation in hypoxia. This effect alone, (independent from any chemoreflex modification), would not be of secondary importance in rehabilitation programs involving both cardiovascular and respiratory patients.

More specific factors, dealing with control of respiration, may be also involved. Powell et al. have reviewed the respiratory changes induced by acute intermittent hypoxia in animal and man (66,67). These include both facilitatory and inhibitory effects. Because the time constants of onset and

decay of these different responses are different, it is conceivable that a specific choice of time for hypoxia and reoxygenation may induce longer lasting modifications in the response to hypoxia. Acute hypoxia increases ventilation, due to both increased tidal volume and breathing frequency; a short term potentiation causes further increase in tidal volume. These effects last for approximately 3-6 minutes, after which, if hypoxia is maintained, a short-term depression reduces the minute ventilation by a reduction in both breathing frequency and tidal volume. It is widely accepted that peripheral chemoreceptors are almost exclusively responsible for the observed initial increase of ventilation; the subsequent ventilatory attenuation seems of central origin rather than an alteration in hypoxic peripheral chemoreceptor discharge (12). In man, the hypoxia-induced increase in ventilation starts falling by effect of short-term depression after about 5 minutes (79). Thus, the choice of sequences of approximately 5 minute hypoxia followed by 5 minutes of reoxygenation (the typical cycle suggested by the Russian groups), seems to match the time required to prevent this central depression. In the conscious dog, if hypoxia is repeated after a similar period of time (in the range of 3-6 minutes), ventilation will tend to increase with each cycle; at the end of repetitive exposures of such duration, ventilation will remain elevated for more than 30 min (12) (long term facilitation). This is only seen after repetitive carotid body stimulation and is not observed following continuous hypoxic exposures or following stimulation of other respiratory afferent inputs (66). However, although it has been described in various animal species, this is not elicited in normal awake human subjects (58).

These findings and considerations do not fully explain the mechanisms whereby HVR is increased by IH, nevertheless they do indicate that a specific time course of events may be necessary to trigger this response. The presence of hysteresis in the responding system and perhaps memory-like effects are probably necessary to maintain it for longer time. Hysteresis could result from a mismatch between the time constants of a physiological process and the frequency of stimulation by periodic hypoxia. Memory-like effects involve plasticity, or changes in structures or fundamental processes in a control system (67). The nature of these processes is to date to a large extent unknown (66).

Hematological effects of IH

Another frequently reported effect of IHT is the increase in hemoglobin (Hb) and RBC production. In the presence of repleted iron stores, the increase in RBC is under the control of Epo, which is secreted into circulation, mainly by the kidneys. Using sensitive radio immunoassay, Eckardt et al. (16) demonstrated a significant increase in Epo after 84 min during 2-hour exposure to 4000m simulated altitude; the Epo half life was

approximately 5.2 hours, peaked after 10 hours and baseline values were reached after 13-26 hours. We have found that 45 minutes of exposure to 12% O₂ is sufficient to significantly increase Epo (8) 2h after termination of hypoxia. Thus, due to relatively fast initial increase, one might expect that relatively few brief exposures to hypoxia may elicit a sustained increase in Epo. The amount of Epo produced appears to be related to the degree of the preceding stimulation (17).

Mild (15.4%O₂) hypoxia administered intermittently (12h/day) or continuously for 7 days to 10 healthy subjects increased Epo levels, serum soluble transferrin receptors and 2,3DPG (48). Continuous hypoxia generated a peak on the first day, then the response remained elevated, but at a lower level, probably due to ventilatory accommodation rising the PaO₂. Instead, IH determined a more steady increase, despite the total exposure to hypoxia was in fact half than during continuous hypoxia. Mice exposed to 14 days of almost continuous hypoxia (22h/day, ~10%), showed a peak after 4 days, but Epo production decreased markedly thereafter (74). The same findings were obtained by Gunga et al., during a period of 10 days at 3600m altitude: both Chilean shift workers and sea-level Caucasian visitors showed, after an initial increase in Epo, a marked drop after 6 days of altitude (33). Conversely, a typical 2-week IHT course (increasing hypoxic time from 15 to 60min, 9.5 to 11% O₂) produced a steady increase in Epo from the fourth day (presumably when total hypoxic time approached 1 hour) (32). This suggests that steady hypoxia tends to induce a rather transitory Epo increase, whereas with IH (if intense enough) the increase seems more steady.

An increase in red blood cell number (RBC), if due to erythropoiesis, is evident only after 3-4 weeks; with shorter observation time this may or may not appear, or may be the result of reduced plasma volume. In our study (2-week IHT, 7), the increase in Hb and RBC nearly reached statistical significance ($p=0.08$), indirectly suggesting that observation for a longer period may have shown a significant change. IH (like exposure to steady hypoxia) is a likely mechanism to stimulate early release of premature red cells, including reticulocytes, which are reported to be increased in nearly all studies, even after short observation times (33, 70). In general, the studies in which hematologic parameters were measured and observational time was long enough, reported an increased RBC production. Unfortunately, in most of these studies plasma volume was not determined. The interactions between erythropoiesis and other systems are complex and still poorly understood. Of interest, in this context, is the suggestion that IH-induced right ventricular hypertrophy, vascular and hematological changes can be reduced or prevented by beta adrenergic-blockade (80). Also the association between exercise and hypoxia, in some IH studies (46,53) may have reduced Epo secretion (71).

Independently from erythropoiesis, increases in HB and RBC in rats could be due to a compensatory splenic contraction during IH. This effect seems to

occur only in response to IH (after at least 4 days of 1h/day 10% O₂) (50). Few data exist on the changes in plasma volume on RBC and hematocrit by effect of IH (53, 70).

Cardiovascular and autonomic effects of IH

IH and blood pressure regulation. Does IHT cause hypertension?

The sleep apnea syndrome, and particularly the obstructive sleep apnea (OSA), can be considered as a "natural experiment" of IH. This syndrome is increasingly being recognized in the clinical field as a potential cause of stable hypertension. Although many factors may be responsible for the sustained increase in blood pressure, such as sleep fragmentation, changes in intrathoracic pressure and respiratory effort to overcome the periodic airway obstruction, IH is considered to play a central role. The clinical importance of this condition has stimulated a large number of experimental studies in rat models of OSA, capable of demonstrating an increase in blood pressure, rather than studying the effects of different patterns of IH per se on blood pressure.

As such, the episodes of hypoxia were extremely short and frequent (even 120/hour, 22); the hypoxia was in general quite substantial (at least for very short periods, in the range of 2-6% O₂), and the total duration of the hypoxic courses was in the range of 6-12 hours/day (3-6 hours of cumulative severe hypoxia). Fletcher et al. observed that the hypertension induced with very short and frequent IH (22) was completely prevented by deafferentation of peripheral chemoreceptors. Several other studies have confirmed that IH (of identical or comparable intensity and frequency) can induce sustained hypertension (4, 22, 23, 24, 31, 51). However, if the hypoxic episodes were more prolonged, less frequent, and/or the total hypoxic time/intensity decreased, the increase in blood pressure was less evident, if not absent (49). Actually, long-term or continuous exposure to lower level hypoxia could even show a protective role against the development of arterial hypertension in spontaneously hypertensive rats (SHR) (35, 64) (Table 1).

In the "fast sequence-severe hypoxia" models (22,23) sympathetic nervous system activity and adrenal sympathetic activity appear to be both necessary, as suppression of each of them separately abolishes the increase in blood pressure (4). Sica et al. found that these models of IH may induce stable activation of different cerebral structures involved in the regulation of sympathetic activity: while at the level of more elementary structures, primarily involved in stimulus recognition and generation of reflexive responses, the frequency of the stimulus presentation may not be an important parameter, only chronic IH activated cortical circuitry (75). Although the rat hypertensive model of IH is different from the IHT as it is

applied to man, these data are nevertheless relevant, as they may show some of the possible mechanisms for long-term adaptation of central nervous system to IH.

Kraicz 1999 et al. (49) have specifically connected the development of hypertension to the short duration of the hypoxic exposures, and the impossibility of vasodilator substances (ANP and BNP, for example) to be able to counteract the induced increase in blood pressure, due to this short time. This suggests that longer cycles of IH may allow some antihypertensive factors to counteract the increase in blood pressure. Looking at Table 1 this seems to be the case. Other studies investigated the role of hypoxia in the protection (!) against blood pressure. In these studies the hypoxia was administered either continuously (35) or in longer periods (64): here hypoxia protected completely (younger SHR) or partially (older SHR) from developing hypertension (35). This is similar to the human response to acclimatization to high altitude, where blood pressure, after an acute increase (due to both neural and adrenal increase in sympathetic activity) drops toward normal values after extended sojourn. In our study in healthy volunteers (7), two weeks of a typical IHT did not change blood pressure.

These observations seem to converge on the idea that IH may or may not develop hypertension on the basis of the frequency, duration, total length and intensity of hypoxic cycles: short and frequent hypoxic episodes maintained for several hours induce hypertension; long exposures to steady hypoxia may even exert a protection against hypertension in specific strains of hypertension prone rats, whereas intermediate training periods of several minutes, as those proposed by the Russian IHT, seems to be neutral in terms of blood pressure control in man (and probably also in terms of left ventricular hypertrophy).

IH and left heart in the rat model

In rat models, left ventricular hypertrophy seems to develop by direct effect of hypoxia, as it was not influenced neither by sympathectomy (despite this prevented hypertension) (51) nor by carotid body denervation (22); instead, it requires a longer period of exposure to IH to become manifest in these models (23). Left ventricular hypertrophy is less evident or delayed further in the models in whom duration of each exposure to hypoxia is longer and intensity reduced. Isolated rat hearts exposed to 30' hypoxia increase RNA synthesis, but this only occurs during the reoxygenation periods (only if energy depletion occurs), thus suggesting a possible mechanism whereby IH may be facilitating ventricular hypertrophy (27).

Table 1. Results of different rat experiments on the effects of intermittent hypoxia on resting blood pressure and other cardiovascular parameters.

Authors	rats	days	total exposure per day (h)	cumulative hypoxia time (h)	# episodes per day
Fletcher (22)	Wistar	35	7	~3.5	840 (~15s)
Fletcher (23)	Wistar	35	6-8	~3	840 (~15s)
		30	6-8	~3	840 (~15s)
		20	6-8	~3	840 (~15s)
Bao (4)	SD	35	6-8	~3	840 (~15s)
Fletcher (24)	F1				
	(SHRxWKY)	35	6-8	~3	840 (~15s)
Greenberg (31)	SD	30	8	~4	480 (30s)
Kraiczi (49)	WKY	70	8	~4	160 (60s)
	SHR	70	8	~4	160 (60s)
Kuwahira (50)	SD	35	1	1	1 (1h)
Obrezchikova (64)	SHR	21	6	6	1 (6h)
	SHR	56	6	6	1 (6h)
Irlbeck (44)	SD	20	8	8	1 (8h)
Henley (35)	SHR 7wk	70	24	24	0 (24h)
	SHR 5wk	70	24	24	0 (24h)

Table 1 (continued).

Authors	lowest O ₂ %	Blood pressure	Heart rate	baroreflex	Hypertrophy	
					LV	RV
Fletcher (22)	3-5	+	=	na	+	=
Fletcher (23)	2-5	+	=	na	+	=
	2-5	+	=	na	=	=
	2-5	~+	=	na	=	=
Bao (4)	2-3	+	=	na	+	=
Fletcher (24)	2-4	+	=	na	+	na
Greenberg (31)	6.5-7	+	=	=	na	na
Kraiczi (49)	6	=	=	na	=	=
	6	=	=	na	+	=
Kuwahira (50)	10	=	+	na	=	=
Obrezchikova (64)	~12	-	=	na	=	+
	~12	+	=	na	=	+
Irlbeck (44)	16 to 10	~-	-	na	na	+
Henley (35)	~14	-	=	na	na	na
	~14	-	=	na	na	na

+: increase; = : no change; -: decrease; na= not available. The numbers in parenthesis are the duration of hypoxic episodes. LV=left ventricle and RV = right ventricle.

IH and right heart in the rat model

Vasoconstriction of the pulmonary circulation is a well-known phenomenon elicited by acute hypoxia. Continuous hypoxia may induce pulmonary hypertension and right ventricular hypertrophy (RVH). Can IHT induce similar unfavorable consequences? There are no studies assessing whether this occurs. However, indirect information can be obtained by rat

models of IH. Nattie et al. (63) evaluated the threshold for intermittent hypoxia-induced RVH in the rat, and found that 2h/day for 42 and 56 days period induced RVH, whereas 1h/day did not. Exposures to IH in cycles of 10 hours per day in the rat can induce important pulmonary vascular remodelling (61) than can be reduced by calcium antagonists. The possibility to develop RVH seems to parallel the duration of hypoxia, and it increases further with longer exposures (44, 62). Marcus et al. (57) argued that transient (seconds to minutes) elevation of right ventricular pressure do not cause RVH, even if the total duration of hypoxia was in a similar range (2h/day). This appears to be confirmed by the studies in which IH was induced by sequences of very brief severe hypoxia for several hours (4, 22,23), in which RVH in general did not occur (Table 1). Also, RVH is not common in OSA. The cause of the RVH seems a combination of the direct effects of intermittent hypoxia on pulmonary vascular resistance (acting both intermittently and by effect of vascular remodelling) as well as the effect of the polycythemia (63). Angiotensin II inhibitor Losartan (44) slightly decreased the gain in RV mass in hypoxic rats, in parallel with a decrease in RV systolic pressure and could completely prevent the increase in RV myocytes. Also IH-induced RVH and pulmonary vasoconstriction has been reported to be counteracted by pre treatment with betablockers (80).

What is the differential role of the increased hematocrit (and viscosity) vs pulmonary pressure on RVH? Studies examining both these factors have shown that IH with very brief duration induce polycythemia without significant RVH (Table 1); if hypoxia is maintained for 1h/day polycythemia develops without RVH (63); longer hypoxia times induce both (63). Increases in red blood cell mass without hypoxia (by cobalt administration) could also induce hypervolemic polycythemia, which seems to contribute to pulmonary hypertension and RVH (80). Thus, both hypoxia-induced pulmonary hypertension and polycythemia may contribute to RVH. To the extent that IH is applied with brief intervals (in the range of several minutes), the risk of RVH due to stable pulmonary hypertension seems negligible in rats.

IHT and autonomic control of heart rate and blood pressure in man

The increase in sympathetic activity is part of the normal response to hypoxia, which, per se, has a depressant effect on the circulation (34). However, excessive sympathetic stimulation may be counterproductive, as in acute mountain sickness (45). Results obtained in rats indicate that minor or no changes in heart rate and baroreflex sensitivity occurred by IH, whereas the effects of IHT on the autonomic nervous system are unknown. IHT increases sensitivity to peripheral chemoreceptors, suggesting that it may also increase sympathetic activity. Alternatively, adaptation to this stimulus may elicit the opposite effect. We therefore evaluated the cardiovascular

autonomic changes occurring by effect of IHT at rest, and during progressive hypoxia (7). Mean values for heart period (RR interval) and systolic blood pressure were obtained at rest, during 1 minute before (baseline) and during the last minute of each rebreathing test. Power spectrum analysis was used to evaluate the fast oscillation in RR interval induced by vagal activity, synchronous with respiration, and the low-frequency component (between 0.03-0.15Hz), which reflect changes in sympathetic activity, in the RR interval and in the blood pressure. Before IHT, progressive hypoxia induced an evident sympathetic activation, as indicated by a decrease in mean RR interval and RR interval variability. The decrease in RR interval variability was due essentially to a decrease in the respiratory sinus arrhythmia, despite the opposite effect of the increasing tidal volume by progressive hypoxia. Conversely, no significant changes were observed in the power of the low-frequency component of the RR interval; thus, in relative terms, there was a progressive increase in the relative proportion of non-respiratory low-frequency oscillations. These findings indicated a decrease in vagal activity, and an increase, at least in relative terms, of sympathetic activity with progressive hypoxia. Systolic and diastolic blood pressure showed a trend toward an increase, but the changes obtained were not significant.

After completion of the 2-week IHT we did not observe significant changes in resting RR interval and blood pressure, although the RR interval was longer (in the training group only) and blood pressure was lower than baseline in both groups. At the end of rebreathing, both mean RR interval and RR interval variability remained higher than before training. This maintained variability was due to a persistence of the respiratory component. The increase remained evident after correction for the increase in tidal volume determined by rebreathing. No significant changes were observed in the modulation of systolic and diastolic blood pressure. In the control group the sham training determined similar responses as before training. Thus, IHT is capable of reducing the effects of hypoxia on the autonomic nervous system, particularly in the parasympathetic component. While this may be the consequence of an increased resistance to hypoxia, it cannot be excluded that habituation of performing the task of breathing in hypoxia would have diminished the associated stress (42).

IH and angiogenesis

Collateral blood vessels supplement normal coronary blood flow and coronary blood flow compromised by coronary artery disease, thereby protecting the myocardium from ischemia. Collateral vessel formation is the result of angiogenesis. Acute tissue hypoxia induces a marked increase in VEGF in the heart (54). However, when the hypoxic stress is chronic and persistent, lasting for days or even months, such as in the case of myocardial hibernation or chronic critical limb ischemia, the angiogenic response to

tissue hypoxia is insufficient. Levy (55) found that the induction of VEGF mRNA, in response to acute hypoxia, was markedly blunted in heart or liver cells previously exposed to chronic hypoxia, and proposed that the lack of compensatory angiogenesis in several clinical conditions is due to a failure to induce VEGF appropriately. At the opposite, in an in-vivo experiment, myocardial IH in pigs hearts was induced by repeated 2-10 min left anterior descending coronary artery occlusions, separated by 20 min of reperfusion for 6 hours. Under these conditions, VEGF expression was significantly augmented in the ischemic territory of the myocardium (three- to fivefold induction). Thus, the effect of intermittent hypoxemia in the myocardium is to significantly upregulate VEGF production. These results suggest that VEGF is a likely mediator in the natural process of ischemia induced myocardial neovascularisation (3). This is confirmed by the evidence that patients with coronary artery disease whose monocytes express lower fold induction of VEGF mRNA in response to chronic hypoxia show a lower degree of collateral circulation in the coronary bed (73). Future studies are needed to further clarify if these results can be transferred to man by a IHT. Finally, these results may support the observation (from muscle biopsies) of increased angiogenesis during intermittent but not chronic hypoxia (40).

Morphometric data on capillarity and muscle fiber size from studies of continuous and intermittent hypoxia

Hoppeler and Desplanches (40) showed that continuous and intermittent hypoxia might have different consequences on muscle fiber size and capillary density.

Continuous hypoxia

All studies report a significant increase in capillary density with exposure to hypoxia. However, this is not due to capillary neoformation, but rather a consequence of the loss in muscle fiber cross-sectional area, the capillary-to-fiber ratio being unchanged (30,39). A possible increase in muscle myoglobin concentration observed with hypoxia exposure (77) would help O₂ diffusion and would further enhance its availability to working muscle mitochondria. However, these results were mostly obtained by exposure to severe hypoxia, where catabolic effects may have been preponderant.

Intermittent hypoxia

In 2 similar studies, muscle biopsies were analysed before and after training periods either in normoxia or under moderate to severe hypoxia. In a study involving one-legged bicycling in either hypoxia or normoxia,

Terrados et al. (77) report capillary to fiber ratio and mean fiber area both slightly (but not significantly) increased after training in hypoxia while they remained unchanged in normoxic training. Desplanches et al. (15) trained 5 subjects for 3 weeks (2hours/day) in severe hypoxia (5500m), and repeated the same protocol 14 months later in normoxia. Training in hypoxia significantly increased average muscle fiber area (+10%), capillary to fiber ratio (+13%) and total mitochondrial volume (+51%). Training in normoxia had no significant effect on any of the morphometric variables analyzed.

Within the limitation of indirect comparisons, made between different experiments and conditions are possible, it seems that continuous and severe hypoxia leads to a state of muscle catabolism in which large quantities of both myofibrillar and mitochondrial proteins are lost and only the capillary network is spared. In contrast, discontinuous moderate hypoxia leads to increase in muscle capillary supply, improved intracellular transfer conditions for oxygen because of an increase in myoglobin concentration and proliferation of mitochondria.

Metabolic effects of IH

There is evidence that chronic hypoxia caused by moderate or high altitude, increases the concentration or activities of enzymes involved in oxidative metabolism. This effect is similar to the endurance exercise training, suggesting that tissue hypoxia is responsible for the changes in mitochondrial density and oxidative enzyme capacity under both conditions (36). In contrast to extreme altitude, which may cause a reduction in the activity of oxidative enzymes (30, 78), at moderate altitude levels (4300m) several studies showed that the oxidative enzymes in the glycolytic pathways, in the Krebs cycle and electron transport chain all increased, whereas no increases were found in lactate dehydrogenase, suggesting that anaerobic metabolism was not potentiated by hypoxia (78). The hypothesis that these effects during physical training can be mediated by some sort of IH is further strengthened by the observation that similar changes in the metabolic activity of skeletal muscles occur in sedentary patients with peripheral arterial insufficiency (37). In these patients, corresponding IH occurs after only slight muscle work, such as walking.

Several experimental works were carried out to sort out the different effects of physical activity, and different hypoxic regimen on oxidative enzymes. Holm et al. (38) exposed rats to various intensities of IH for 3 hours/day for 1-4 weeks. Before and after muscle biopsies were taken from the vastus lateralis for determinations of succinic oxidase activity, rate of incorporation of glucose into glycogen, lipids, lactate, and tissue CO₂, shown previously to be elevated in patients with arterial insufficiency (37). The most severe degree of IH (5% O₂) decreased pH and CO₂, whereas IH at

10% O₂ increased pH and decreased CO₂. All enzyme and metabolic turnover measures were decreased by severe IH, increased by 8% or 10% O₂ IH, and unchanged by mild IH and in control rats. The changes induced during 8% and 10% O₂ IH were also followed for 4 weeks: after that time they observed significant increase in Hb concentration; phospholipid concentration of the vastus lateralis decreased and protein concentration increased significantly; succinic oxidase activity and all rates of incorporation of glucose-carbon increased whereas an inverse trend was seen for lactate. This study suggests that IH may be sufficient to cause changes in the skeletal muscle tissue similar (at least qualitatively) to those of prolonged stay at altitude, and similar to those seen after physical training. However IH with extreme hypoxia can reverse the results, leading to catabolic effects, similarly to what has been found in men during exposure to extreme altitude (40, 78). Terrados et al. (77) in a study involving one-legged bicycling reported a significant larger increase in citrate synthase (+30%) in the leg trained in hypoxia (2300m) than in the leg trained in normoxia. Moreover, myoglobin increased in the leg trained under hypobaric conditions while it decreased in the leg trained under normobaric conditions. Again, this study shows that hypoxia is a common stimulus for development of the oxidative capacity even during normoxic exercise, and the effects observed should be mainly ascribed to the hypoxia regimen. In this study the time of hypoxic exposure was limited to 30min for 3-4 sessions per week, and the hypoxia was comparable to an altitude of 2300m. The increase of mitochondrial enzymes seems to be preceded by acute alterations in energy metabolites due to IH. This enzyme adaptation allows the muscle tissue to maintain a lower anaerobic metabolism and a better energy state during exercise (19).

Oxidative stress and intermittent hypoxia

The oxidative stress is strongly implicated in the pathophysiology of important diseases, including diabetes, atherosclerosis, cancer and aging, and may influence severe acute conditions such as myocardial infarction and cardiac arrhythmias. The Russian literature reports many effects of IHT on protection against oxidative stress. In the western literature growing evidence now seems to converge on similar concepts. Brief episodes of ischemia and reperfusion increase myocardial tolerance to a subsequent sustained period of ischemia. The late phase of protection, 24 hours after the initial preconditioning, involves transcription of mRNA and subsequent synthesis of certain protective proteins, including antioxidant proteins.(14) The reduction of infarct size in the preconditioned rat myocardium seems associated with the increase in Mn superoxide dismutase activity (41). Isolated rat myocytes, preconditioned either with two cycles of 5 minutes of anoxia and 5 minutes of reoxygenation or with exogenous superoxide anion

(O_2^-), demonstrated remarkable resistance to both early and late subsequent anoxia; this was characterized by increased superoxide dismutase activity (81). These results suggest that a burst of oxygen free radicals generated during the initial periods of brief, repetitive anoxia increases myocardial antioxidant activity 24 hours later and that it contributes to the late cardioprotective effect of preconditioning. There is also evidence that a similar course of intermittent anoxia (10 minute sequences) reduces oxygen free radicals formation during reoxygenation in rat hepatocytes. (26)

Exercise tolerance by IH

Effect of IH on Exercise tolerance

The effects of chronic hypoxia on blood oxygen transport capacity, erythropoietic response and the increase in Hb affinity for oxygen are well known. Athletes at altitude train at lower VO_2 max, which reduces the benefits obtained from altitude acclimatization. The most effective technique for increasing endurance seems to be to live at altitude and train at sea level (53) but this may not be always easily applicable. In theory, IHT could be a practical method to obtain a similar goal. Only few studies evaluated the effect of IH alone (without associate training) on exercise performance.

Rodriguez et al. (70), after 9 days of staying 3 to 5 hours at a simulated altitude of 4000 to 5500m, found no differences between the exercise performance of subjects who did train during altitude exposure, and subjects who did not. Pooling all data together, they observed an increase in (sea-level) exercise capacity, maximal pulmonary ventilation, and no difference in maximal VO_2 . In addition, they found a reduction in lactate production at each step of exercise, and an increase in RBC and Hb and reticulocytes, together with increase in plasma osmolality, and slight non-significant increase in plasma viscosity. Subsequently, the same group (13) repeated the same IH protocol, but without any training involved and for a period of 17 days in 6 elite climbers. They again found a right shift of lactate-load and heart rate-load curves, and increase in hematological parameters. They concluded that the lower lactate accumulation, during the incremental exercise test performed after IH, reflected an enhancement of aerobic endurance capacity, which was confirmed by an increase in VO_2 , VCO_2 , VE/VO_2 and VE/VCO_2 , V_t and VE , and Fr in relation to the blood lactate concentration during the maximal incremental tests.

Can IHT alone affect physical performance?

While these results indicate that IH alone may be able to enhance oxygen transport as well as aerobic endurance capacity, the question remains as to

whether a simple course of typical IHT can induce comparable results. In collaboration with the Moscow Hypoxia Medical Academy, the Department of Sport Science of the University of Innsbruck has been evaluating this possibility.

In a first study (10), they randomly exposed 28 young healthy subjects to IHT (20 IHT sessions in 4 weeks, each consisting of 3 to 7 exposures to 11%-9% O₂ of 3 to 6 min, with 3 to 5 min normoxic intervals) or to sham training (same, but breathing air) in a double blind fashion. Five days after completion of IHT, significant differences between IHT and placebo group were found at the submaximal work load of 150 watts for exercising heart rates and minute ventilation. Subgroup analyses of stroke volume determinations indicated that lower heart rates were compensated by an increased stroke volume. The double product was also significantly reduced at 150 watts in the IHT group. One month after completing the breathing program the changes were still evident (though not significant). They suggested that the repeated application of short term hypoxia by hypoxic air breathing or altitude exposure (9) reduces sympathoadrenergic responses to submaximal exercise. Since the reduction of such responses enhances exercise tolerance and reduces mortality in patients with coronary artery disease (CAD) (29) and because acute hypoxia is well tolerated by those patients (20) they hypothesized that IHT might a potential therapeutic use. For this purpose they subsequently compared the effects of IHT and ambient air breathing on exercise tolerance and aerobic capacity in elderly men with and without previous myocardial infarction (11). Sixteen elderly men (8 with prior myocardial infarction and 8 controls, aged 50 – 70 years) were randomly assigned to placebo or IHT in a 3-week protocol (as in 10). After this period, they found significant increases in peak VO₂ and minute ventilation in the IHT vs. placebo group. After IHT, at maximal workload oxygen saturation and minute ventilation were higher, whereas blood lactate concentrations were lower. At the same submaximal work loads (1.5 watts/kg) heart rates and double product were decreased in the IHT group. Hb was significantly increased after IHT (however, no reticulocytes or plasma volume were measured). Subgroup analyses revealed all changes to be more predominant in patients with CAD. IHT was well tolerated even in CAD patients, and no relevant side effects occurred. After a 3-week IHT program, exercise tolerance and aerobic capacity were thus increased, particularly in CAD patients. These effects may mainly be due to an increase in oxygen transport capacity and reduced blood lactate accumulation, with concomitant reduced sympathetic stimulation by similar workloads. These first results in cardiac patients are to be considered encouraging, even if re-treatment intervals to maintain these effects remain to be assessed, and need further investigation.

IH and adaptation to high altitude and training for athletic performance

Hypoxic training as a means for adjusting to oxygen lack may be of value to improve competitive performance at altitude or at sea level, or to improve acclimatization for work or climbing at altitude by decreasing symptoms of mountain sickness. IH has been an integral part of the strategy of climbers to adapt to high altitude, and several groups have studied the physiology of IH as a means of pre acclimatization (67). Training for athletic performance at low or high altitude seems to benefit from IH (53), and oxygen enrichment during night time (56) seems to improve working at altitude. In general, some of the favorable effects reported are similar to those that have been reported for IHT (improvement in oxygen transport and increased aerobic capacity), however, so far there are no studies examining the effects of a standard IHT for these purposes, so no direct comparison is possible. In a recent newsletter, Houston (43) concluded that current evidence, for the purpose of using hypoxic training to acclimatization, suggests: "1) training to compete at any altitude is best accomplished by sleeping high and training low. There may be a break point around 1500m, above which this precept weakens; 2) acclimatization for ascent to very high altitude is best accomplished by exposure to gradually higher altitudes (i.e. to gradually decreasing ambient oxygen pressure); 3) the effect of IH in preventing mountain sickness has not been proven; and 4) the beneficial effects of IH in accelerating acclimatization are suggestive but require further rigorously controlled study". At this time these same conclusions seem to apply to IHT as well. To what extent experience with the current or modified IHT could modify these conclusions is matter for future investigation.

CONCLUSIONS

IH seems capable of inducing a large variety of effects at different levels, in the oxygen transport and energy utilization, in the respiratory, autonomic, cardiovascular and perhaps even in other systems; some of these effects, might be potentially beneficial in specific physiologic or pathologic conditions. The effects of intermittent vs chronic hypoxia are often considerably different; one striking example is the possibility to induce stable arterial hypertension or even protect from it (in specific rat models) by different IH protocols.

Remarkably, when comparing the different effects of IH in the studies appeared in the western literature, there seems to be a converging suggestion that a course of several relatively short (3-10 minutes) exposures to moderate hypoxia (9-12% O₂), interrupted by the same amount of time, for a

total of 30-60min hypoxic exposure per day for approximately 2 weeks, may have several interesting and potentially useful applications, without unfavorable side effects. This "ideal" protocol corresponds to what has been in fact suggested in the Russian literature as a standard IHT. Analysis of the western literature suggests that a similar IH protocol would increase HVR, increase Epo production (and red blood cell count later), increase aerobic capacity and exercise performance, moderate sympathetic activation and preserve parasympathetic activity during the hypoxic exposure and physical exercise, induce angiogenesis, protect (isolate cells) against free-radical injury, while avoiding unfavorable effects such as systemic hypertension, left and right ventricular hypertrophy.

Clearly, many of these effects can be only speculated by comparing highly different experimental conditions (spanning from isolated cell preparations to rat models, and to man in only few instances). A large amount of study is necessary to further clarify all the complex dynamic interactions involved in different IH protocols and in different systems, whose importance starts being appreciated only in the present years. If the effects of IH could be better known in man, it is likely that manipulation of IH protocols could have practical applications not only in sports and mountaineering, but also in clinical medicine (eg in myocardial preconditioning and rehabilitative programs in cardiac respiratory, and autonomic patients).

Although the few IHT studies appeared in the western literature have so far confirmed the hypotheses formulated by Russian authors, at this stage this technique can be considered of interest but still largely to be explored for its mechanisms, potentials and limitations. In particular, studies on phenotype expression of the various factors involved in the specific response to intermittent vs chronic hypoxia are lacking, but likely great progress will be made in this field in short time.

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Chapter 26

Gene transfer and metabolic modulators as new therapies for pulmonary hypertension

Increasing expression and activity of potassium channels in rat and human models

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Abstract: Chronic Hypoxic Pulmonary Hypertension (CH-PHT) is characterized by pulmonary artery (PA) vasoconstriction and cell proliferation/hypertrophy. PA smooth muscle cell (PASMC) contractility and proliferation are controlled by cytosolic Ca^{++} levels, which are largely determined by membrane potential (E_M). E_M is depolarized in CH-PHT due to decreased expression and functional inhibition of several redox-regulated, 4-aminopyridine (4-AP) sensitive, voltage-gated K^+ channels (Kv1.5 and Kv2.1). Humans with Pulmonary Arterial Hypertension (PAH) also have decreased PASMC expression of Kv1.5 and Kv2.1. We speculate this “ K^+ -channelopathy” contributes to PASMC depolarization and Ca^{++} overload thus promoting vasoconstriction and PASMC proliferation. We hypothesized that restoration of Kv channel expression in PHT and might eventually be beneficial. **Methods:** Two strategies were used to increase Kv channel expression in PASMCs: oral administration of a metabolic modulator drug (Dichloroacetate, DCA) and direct Kv gene transfer using an adenovirus (Ad5-Kv2.1). DCA a pyruvate dehydrogenase kinase inhibitor, promotes a more oxidized redox state mimicking normoxia and previously has been noted to increase K^+ current in myocytes. Rats were given DCA in the drinking water after the development of CH-PHT and hemodynamics were measured ~5 days later. We also tested the ability of Ad5-Kv2.1 to increase Kv2.1 channel expression and function in human PAs ex vivo. **Results:** The DCA-treated rats had decreased PVR, RVH and PA remodeling compared to the control CH-PHT rats (n=5/group,

$p < 0.05$). DCA restored Kv2.1 expression and PASMC Kv current density to near normoxic levels. Adenoviral gene transfer increased expression of Kv2.1 channels and enhanced 4-AP constriction in human PAs. **Conclusion:** Increasing Kv channel function in PAs is feasible and might be beneficial.

Key words: adenovirus gene therapy, Kv1.5, Kv2.1, redox state, dichloroacetate and metabolic modulators

INTRODUCTION

Pulmonary hypertension (PHT) remains a major cause of morbidity and mortality in humans. (2) Therapies for PHT, though improving, are still expensive and often are ineffective. (2) Recent studies of both human and experimental PHT have noted an abnormality of cellular electrophysiology that may have importance in the pathogenesis of this syndrome. Specifically, that K⁺ channel function and expression is depressed in pulmonary artery smooth muscle cells (PASMC) from patients and animals with various forms of PHT (24, 28, 31, 34). In human pulmonary arterial hypertension (PAH), the basis for this reduction in expression and whole cell K⁺ current (I_K) is unknown. Somewhat more is known about changes in K⁺ channels in experimental PHT.

In rats with chronic hypoxic PHT (CH-PHT), PASMCs have reduced K⁺ current density (Figure 1). This depolarizes membrane potential (E_M) to a level where L-type Ca⁺⁺ channels are active (24, 28) and thus elevates cytosolic calcium concentrations (Ca^{++})_i promoting vasoconstriction and cell proliferation. (23) CH-PHT and human PAH are associated with impaired expression of specific Kv channels, notably Kv1.5 and Kv2.1 (24, 30, 31, 34). These Kv channels are involved in the control of PASMC membrane potential and the mechanism of hypoxic pulmonary vasoconstriction (HPV). (6) The mechanism for Kv channel downregulation in PAH is unclear, but recent work suggests that, at least in CH-PHT, it may relate to the reduced redox state induced by chronic hypoxia (CH). (24) We have previously shown that reducing agents inhibit whilst oxidants activate I_K in PASMCs. (25) Hypoxia creates a reduced redox state in the lung, diminishing production of activated O₂ species within seconds (4) and increasing reduced glutathione levels, within minutes. (24)

We hypothesize that downregulation of Kv channel function and expression is causally related to the development and maintenance of CH-PHT and possibly PAH. We sought to enhance expression and function of Kv channels using dichloroacetate (DCA), a metabolic modulator, which has been shown to increase I_K in cardiac myocytes from infarcted rats (27). DCA inhibits the mitochondrial pyruvate dehydrogenase kinase (PDK) (29) and

by increasing the pyruvate / lactate ratio, might promote an oxidized state.(8, 9) Thus, we speculated that DCA would reverse the reduced redox state in the CH-PHT rats and might thereby enhance the activity and expression of Kv channels and reverse CH-PHT, mimicking the benefits of a return to normoxia (Figure 2).

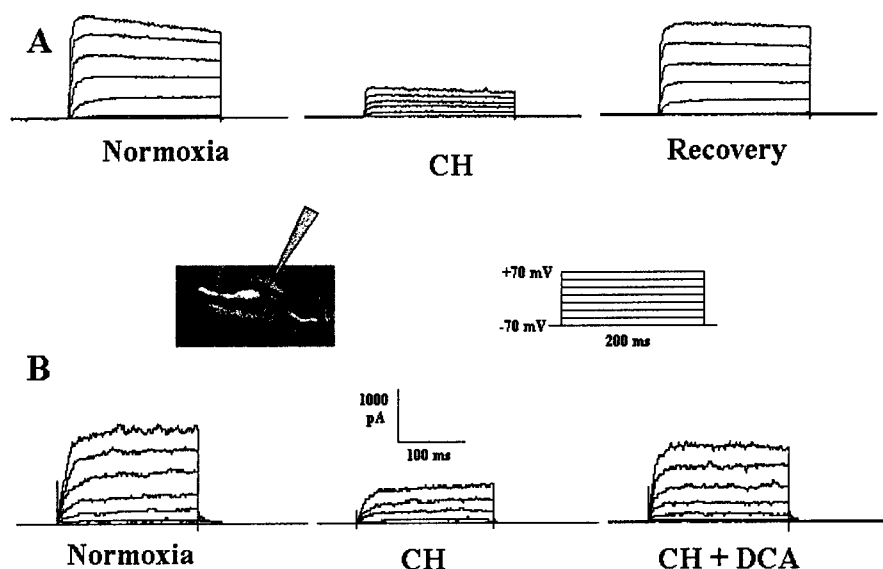


Figure 1. Chronic hypoxia decreases K⁺ current in PASMCs and this can be reversed by DCA. Representative K⁺ current measured using whole cell patch clamp technique. A) CH decreases K⁺ currents within 2 days of CH and this recovers when rats are returned to normoxia for 1 week to normoxia. B) DCA in the drinking water restores K⁺ currents to near normoxic levels despite continued hypoxia.

We also hypothesize that adenoviral gene transfer with a vector containing the gene for Kv2.1 channels (e.g. Ad5-Kv2.1) can directly augment Kv2.1 channel expression and alter PA vascular tone in human PAs. The ultimate goal of this research is to test the feasibility and utility of altering the expression and function of K⁺ channels as a treatment for human PHT.

METHODS

CH-PHT

Age and weight matched male Sprague-Dawley rats were randomized to either the normoxia, CH or CH plus DCA group (CH-DCA) (n=5 for each).

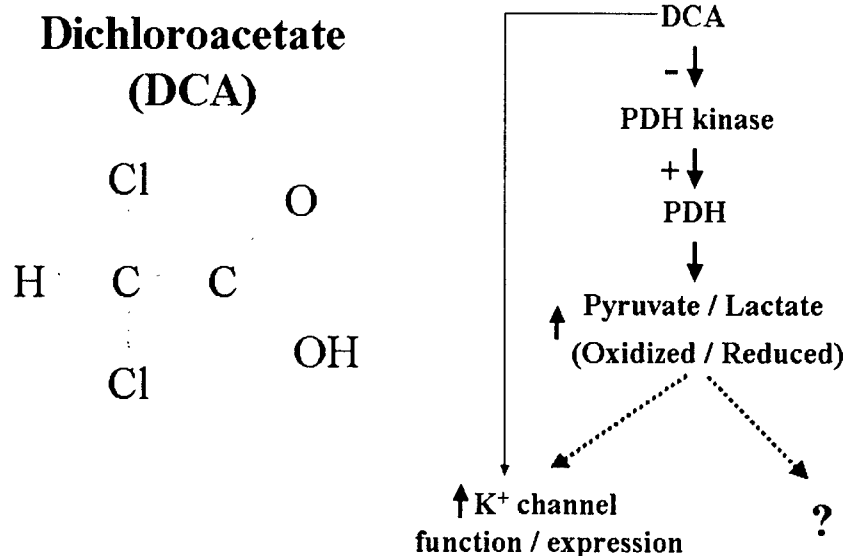


Figure 2. Schematic diagram illustrating the possible mechanism by which DCA might alter K⁺ channel function and expression. The "?" refers to the possibility that there are potentially mechanisms in addition to the chronic change in cytosolic redox state by which DCA may work. These include the new finding that DCA acutely increases IK (Figure 5).

CH-PHT was created by exposing the rats to normobaric hypoxia (10% O₂), as previously described (5). The water for the CH-DCA group contained DCA (0.75g/l) and rats ingested, ~ 70 mg/kg/day. Exposure to DCA started on day10 of CH (n=5) to test for reversal of CH-PHT.

Hemodynamic Measurements

Beginning on day 14 of the protocol, the rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Rats were ventilated with room air (Harvard Apparatus, Holliston MA, tidal volume 2.5cc, respiratory rate 80/min, PEEP 2.5cmH₂O). Systemic blood pressure was measured from the left carotid artery, as previously described (21). PAP was measured using a 1.4 French Millar® micro-sensor (Millar Instruments, Houston, TX). Left ventricular end-diastolic pressure (LVEDP) was also measured by retrograde

cannulation using this catheter. Cardiac output (CO) was measured by the Fick method (15). Pulmonary vascular resistance (PVR) was calculated as: mean PA-LVEDP/CO. The RV/(LV+septum) ratio, a measure of right ventricular hypertrophy (RVH), was measured as previously described.(5) A section of lung was also formalin-fixed for histology studies, as previously described (5).

Electrophysiology

Freshly dispersed PASMC were isolated and studied using whole-cell, amphotericin perforated-patch technique, as previously described.(6, 20) The effects of hypoxia were studied by switching between solutions bubbled with normoxic gas ($PO_2=140\text{mmHg}$, pH 7.40) or hypoxic gas ($PO_2=40\text{mmHg}$, pH 7.40). Cells were voltage clamped at a holding potential of -70 mV and currents were evoked by steps from -70mV to +50mV using test pulses of 200 millisecond duration at a rate of 0.033 to 0.1 Hz. Data were recorded and analyzed using pCLAMP 6.02 software (Axon Instruments, Foster City, CA), as previously described.(6, 20) Whole-cell currents for each cell were divided by the cell's capacitance, giving a measure of current density.

Immunoblotting was performed on pooled 4th division PA samples (n=3 rats/group; 25 μg protein/group), as previously described. (19) Antibodies to K^+ channels were purchased from Alomone (Jerusalem, Israel).

Construction and Purification of Recombinant Adenovirus Encoding Kv2.1

The recombinant adenovirus encoding K_v 2.1 was prepared as described by Hu *et al.* using Adeasy-1 and pAdTrack-CMV plasmid obtained from Dr. Vogelstein's laboratory (16). Adeasy-1 is an adenoviral backbone vector, which contains adenovirus (serotype 5) genomic DNA with E1 and E3 deleted. Since E1 domain is important for viral replication and E3 region encodes proteins involved in evading host immunity, deletion of these domains not only provides space for insertion of a transgene but also renders the virus incapable of replication. While Adeasy-1 acts as the adenoviral backbone, pAdTrack-CMV is a shuttle vector, which carries the gene of interest. pAdTrack-CMV is a plasmid which contains kanamycin resistance gene and two cytomegalovirus (CMV) promoters (one promoter located upstream of green fluorescent protein and the other promoter lying upstream of the region designed for the insertion of the gene of interest). When pAdTrack-CMV and Adeasy-1 are co-transformed into BJ5183 cells (a strain of *E.coli* with highly efficient homologous recombination machinery), pAdTrack-CMV carrying the gene of interest undergoes homologous recombination with the adenoviral backbone resulting in a plasmid that

contains much of the adenoviral genome in addition to the gene of interest. Subsequent viral production and replication is carried out in the packaging cell line, HEK 293 cells, which provides E1 and E3 proteins in trans. Multiple rounds of replication and isolation of adenovirus are carried out to obtain high titres of the adenovirus.

For the production of adenovirus carrying the Kv 2.1 gene (Ad5K_v2.1), a 2609 bp cDNA fragment encoding the open reading frame of the rat Kv 2.1 channel was excised from its original pBK-CMV plasmid (kindly provided by Dr. K Takimoto, University of Pittsburgh) using restriction endonuclease Not I and Sal I. The K_v 2.1 cDNA was subsequently ligated into the Not I and Sal I sites of pAdTrack-CMV. The resultant pAdTrack-CMV K_v 2.1 construct was linearised with a Pme I restriction endonuclease digest, transformed together with supercoiled adenoviral vector Adeasy-1 into BJ5183 cells and plated on LB plates containing kanamycin. Subsequent colonies were isolated, grown in liquid LB kanamycin media and the plasmid DNA was purified using plasmid purifying columns (Qiagen). Restriction endonuclease digests using Pac I was performed on the isolated plasmid DNA to select for the plasmid containing K_v 2.1 cDNA within the adenoviral DNA. The selected plasmid was amplified, purified, linearised by Pac I endonuclease digestion and transfected into HEK 293 cells using LipofectAMINE reagent. Five to seven days after transfection, plates that demonstrated complete cell lysis were collected and analyzed for K_v 2.1 cDNA using PCR. Upon confirmation of the K_v 2.1 cDNA in the viral genome, multiple rounds of Ad5K_v2.1 replication were performed in HEK 293 cells to obtain a high titre viral stock. The resulting virus carrying Kv2.1 cDNA was isolated, precipitated and concentrated by discontinuous CsCl gradient. Viral titres were determined by infection of a monolayer of HEK 293 cells with serial dilution of recombinant virus and subsequent agarose overlays. The final viral titre obtained for Ad5K_v 2.1 was 1.5×10^9 pfu/ml.

Statistics

Values are expressed as the mean \pm SEM. Intergroup differences were assessed by a repeated measures ANOVA (for patch clamp data) or factorial ANOVA, as appropriate, with post hoc analysis using a Fisher's probable least significant differences test (Statview 4.02, SAS Institute, Cary, NC). A $p < 0.05$ was considered statistically significant.

RESULTS

CH-PHT

Exposure to chronic hypoxia for ~2 weeks resulted in the development of significant PHT as shown by the increased PA pressure (Figure 3), PVR, RVH and PA remodeling, i.e. medial hypertrophy and neo-muscularization of the resistance pulmonary arteries (Table 1, Figure 4C). The PA pressures and PVR in CH-DCA rats were significantly decreased compared to the CH rats and were similar to values in normoxic rats. The systemic pressure, left ventricular end-diastolic pressure and cardiac output did not differ statistically amongst groups (Figure 2) (LVEDP: 9-10 mmHg for all groups, data not shown). The RV/(LV+septum) ratio was also significantly decreased in the CH-DCA compared to the CH rats, suggesting reversal of RV hypertrophy (Table 1).

CH-PHT Electrophysiology

I_K was significantly decreased in the PASMC from the CH rats but was significantly greater in the CH-DCA rats, almost back at the level of the normoxic rats (Figures 1B and 4A and B). The I_K in PASMCs from normoxic control rats was inhibited ~ 50% of by 4-AP (5mM), indicating that at least half the current it is conducted by K_v channels (data not shown). In the CH rats, the I_K was reduced in magnitude (Figure 1) and was not further inhibited by 5mM 4-AP (data not shown). Similarly, whereas acute hypoxia significantly inhibits I_K in normoxic PASMCs this O_2 -sensitivity is lost in but not CH PASMCs and is partially restored in PASMCs from CH-DCA rats (data not shown). Finally, acute administration of DCA, at the low doses that are not normally associated with metabolic modulation (1 μ M), rapidly increased the I_K in PASMCs from CH (Figure 5).

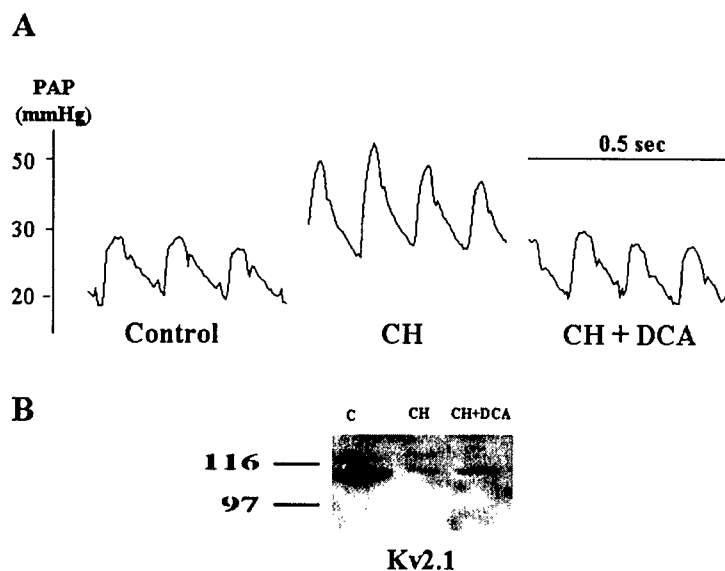


Figure 3. DCA Reverses CH-PHT and Increases Expression of Kv2.1. A Representative high fidelity trace from the PA in the 3 rat groups studied. Chronic administration of DCA in the drinking water reverses the rise in PA pressure caused by chronic hypoxia. B Immunoblotting data on isolated PAs (pooled from 3 rats in each group). CH causes a decrease in the expression of Kv2.1. Chronic treatment with DCA significantly reverses the downregulation of Kv2.1. Using competition assays, we have recently shown that the Kv2.1 antibodies used are specific, at least in rat pulmonary vessels (20).

Table 1. Mean hemodynamic data on the 3 rat groups studied (regression protocol).

n	PVR	Systolic RVP mmHg	Diastolic RVP mmHg	Mean RVP mmHg	CO ml/min	RV/(LV+S)
Normoxic, 5		41.9 ± 2.7	7.0 ± 1.3	17.0 ± 1.3	226 ± 57	.273 ± .019
CH, 5		74.2 ± 2.7	5.3 ± 0.7	28.8 ± 1.5	151 ± 22	.530 ± .024
CH-DCA, 5		50.5 ± 3.5	7.6 ± 1.1	22.7 ± 1.0	206 ± 42	.410 ± .024

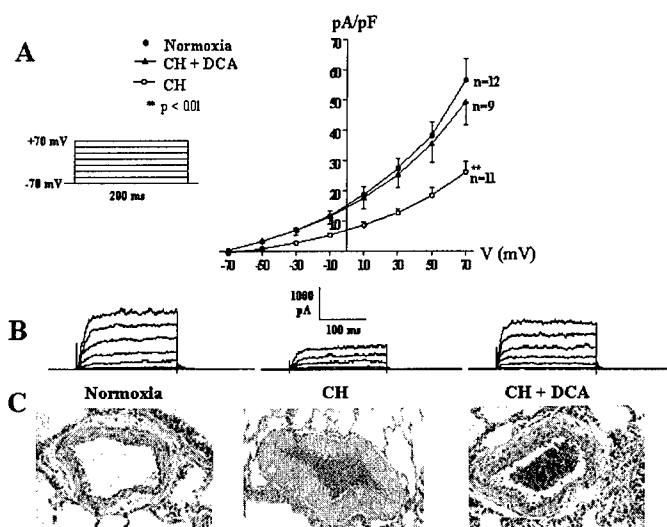


Figure 4. Mean hemodynamic data on the 3 rat groups studied (regression protocol). DCA reverses the increase in PA pressure, PVR and RV hypertrophy (RV/LV+septum) that is caused by chronic hypoxia, while it has no significant effects on the CO. NS: nonsignificant. **Figure 4.** DCA restores K⁺ current and reverses remodeling of PAs caused by CH. **A)** Mean data of the potassium current density (current amplitude / cell capacitance) plotted against the voltages studied in our patch clamp protocol (shown on the left). Chronic treatment with DCA causes a reversal of the decrease in the current density caused by chronic hypoxia, across the whole membrane potential spectrum studied. **B)** A representative raw trace of the IK from each group studied is shown at the bottom. **C)** Histology (H&E stain) of medium sized PAs from the 3 rat groups studied at 25X magnifications. DCA decreases the PA remodeling (medial hypertrophy) caused by chronic hypoxia.

Immunoblotting

Whilst Kv1.5 (24) and Kv2.1 (Figure 3B) are significantly downregulated in the CH, the expression of other Kv channels and the large conductance calcium-sensitive K⁺ channel (BK_{Ca}) channels was unaltered (not shown). DCA partially reversed the downregulation of Kv2.1 (Figure 3B).

RESULTS HUMAN PA KV GENE TRANSFER

We were able to construct an Ad5-Kv2.1 vector was able to effectively transfer Kv2.1 to Chinese Hamster Ovary cells (CHO, Figure 6). Preliminary work indicates that incubation of Ad5Kv2.1 virus at a multiplicity of infection (MOI) of 75 for 48 hrs was sufficient to achieve a 90% infection of CHO cells (Figure 7). Although higher doses of virus (MOI of 375 and 750) were correlated with higher infection rate, these doses were also associated with increased viral toxicity. Uninfected CHO cells lacked Kv current whereas those infected for 24-48 hours developed a large, 4-AP sensitive Kv current (7B). Increasing doses of virus resulted in increasing expression of GFP in CHO cells (Figure 7C). The vector was also able to infect human PA's ex vivo (Figure 8). Infection could be detected noninvasively by examining the PA ring using confocal microscopy (excitation at 488nm). The Kv2.1 transgene was translated in to increased mRNA and protein in the PA (data not shown). Although the results are very preliminary, it appears that the constrictor response to 4-AP was enhanced in rings infected with Ad5-Kv2.1 (Figure 8B).

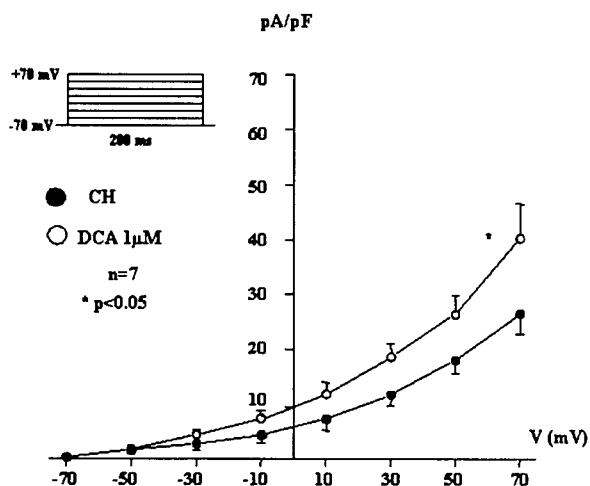


Figure 5. DCA Increases IK in PASMICs from chronically hypoxic rats. Mean data of the effects of DCA solution (1μM) on the PASMIC IK (current density). When given acutely on PASMIC, low dose DCA increases IK in the CH PASMICs within 5 minutes but it has no effect on the normoxic and CH-DCA groups (not shown).

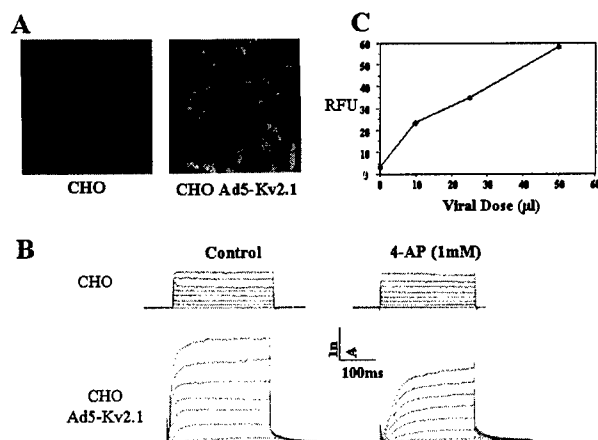


Figure 6. Ad5 Vector construct. In the upper panel is the adenoviral genome, showing the E1 insertion site for the genes of interest. In the middle panel is a vascular myocyte 24 hours after infection with Ad5 containing genes for Kv2.1 and GFP, showing GFP fluorescence. In the lower panel is RT-PCR showing that a smooth muscle cell line infected with the Ad5 KV2.1-GFP virus expressed much more Kv2.1 mRNA than control uninfected cells or cells infected with Ad5 containing only the GFP gene.

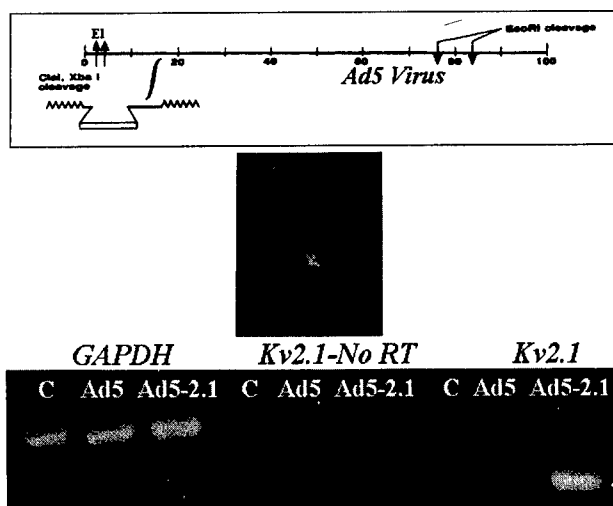


Figure 7. Effect of increasing dose of virus on the expression of GFP in CHO Cells A) Confocal imaging of CHO cells without (minimal fluorescence) or with Ad5-Kv2.1 infection for 48 hours (marked green fluorescence). The infected cells appear bright gray in this black and white image. Imaging was done at 488 nm. B) Normal CHO cells lack a Kv current and are insensitive to 4-AP (upper row). Kv2.1 gene transfer confers a Kv current that is sensitive to 4-AP to CHO cells (lower row). C) The greater the dose of virus administered to CHO cells the greater the RFU (relative fluorescent units). This indicates GFP is a useful marker of the degree of infection and gene transfer.

DISCUSSION

Pathological findings consistent with PPH were first described in autopsy specimens a century ago (26), although the first antemortem diagnosis was not made until 1951 (14). The pulmonary arteries in PPH are characterized by intimal fibrosis, medial hypertrophy, adventitial proliferation, and obliteration of small arteries (Figure 1). Although the recent description of a gene mutation(s) in familial PPH is an important advance in our understanding of this condition (13), there are many unanswered questions. It is unclear how the mutations in the bone morphogenetic protein receptor-II gene causes PPH and what other abnormalities may occur in association with these mutations. We are interested in the hypothesis that PHT may be caused or exacerbated by a deficiency in the expression and function of one or more K⁺ channels. (2) In humans with PAH, Kv1.5 mRNA levels are reduced in PASMCs (34). This downregulation of Kv1.5 is associated with inhibition of K⁺ current, membrane depolarization and elevation of (Ca²⁺); (30, 34). Thus, decreased expression or function of K⁺ channels in PASMC in PPH patients could initiate and/or maintain pulmonary vasoconstriction and play a role in the pathogenesis of PAH. Less is known about Kv2.1, although it seems more important than Kv1.5 in setting Em in rat PASMCs (6). It is fascinating that Kv2.1 is also inhibited by the anorexigen, dexfenfluramine (32), a weight loss drug that is associated with development of PAH (1). We have postulated that there is a causal role for K⁺ channel deficiency in PAH (33), much as occurs with mutations of K⁺ channels in cardiac myocytes of patients with Long QT syndrome (3). Major questions remain, including whether the loss of specific K⁺ channels is a cause or a response to PAH and which specific K⁺ channels are involved.

The current preliminary studies identify two new ways of augmenting K⁺ channel expression and function. This preliminary study suggests that DCA reverses established CH-PHT (Table 1). DCA's beneficial effects are associated with electrophysiological effects, consistent with recovered Kv channel function and expression (Figure 1). When given acutely, DCA increases I_k in freshly isolated PASMC from rats with CH-PHT (Data not shown). When given chronically, DCA reverses the chronic hypoxia-induced downregulation of specific Kv channels, such as Kv2.1 (Figure 1). We propose that the effects of DCA treatment on the function and expression of Kv channels are responsible for the ability of this drug to reverse CH-PHT (Figure 9). Hemodynamic assessment indicates systemic arterial pressures remains unchanged (Table 1). DCA's effects on the left ventricular performance have been studied extensively with conflicting

results, (10, 18) but its effects on the pulmonary circulation have never been studied before.

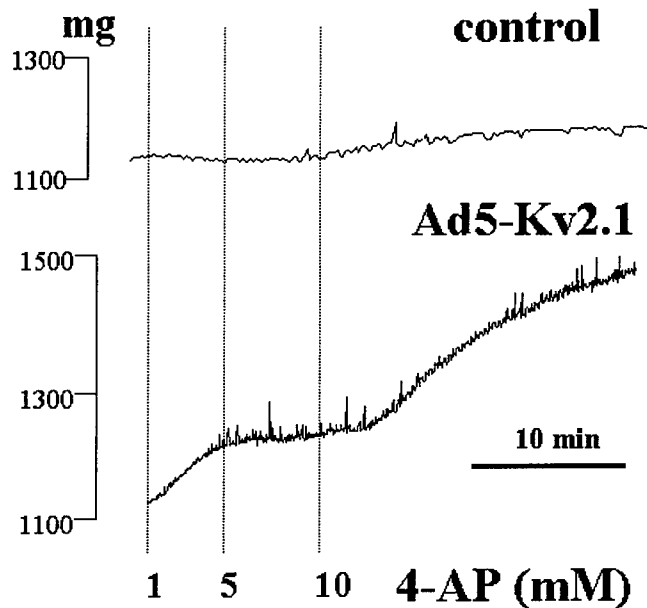


Figure 8. Successful transfer of functional Kv2.1 channel gene to human PA. A representative tracing showing the constrictor response to 4-AP, a Kv channel blocker, in PA rings from a single PHT patient without gene transfer (upper trace) versus following gene transfer (lower trace). The overexpression of Kv2.1 confers enhanced constriction in response to 4-AP.

The data support the concept that Kv channel inhibition and downregulation might be etiologically related to the development of CH-PHT. Smirnov et al first showed that in PASMCM from rats with CH-PHT the function of Kv channels is suppressed and PASMCMs are depolarize (28). It was later shown that CH-PHT is associated with a downregulation of the expression of several Kv channels, such as Kv1.2 and Kv1.5 (31) as well as Kv2.1.(24) Yuan et al have also found a specific downregulation of Kv1.5 in PAs from patients with Primary Pulmonary Hypertension (PPH). (34) Despite all these studies, uncertainty remains as to whether these changes in Kv function and expression are etiologically linked to the PHT or are secondary phenomena or “markers” of the disease. Our preliminary finding that DCA reverse established CH-PHT by reversing the changes in both the

function and expression of Kv channels, supports a potential causal role for K^+ channel deficiency in the pathogenesis of this form of experimental PHT.

How is DCA increasing I_K ? We show that DCA upregulates expression of Kv2.1 (Figure 1), a channel which participates in determining membrane potential in rat PASMCs.(6) Although immunoblotting is only a semiquantitative method, the downregulation of Kv channels in CH-PHT (Figure 1) confirms previously published data.(24, 31) These are likely not nonspecific effects since other channels were not affected and indeed there was a trend for upregulation of the expression of BK_{Ca} channels (data not shown).

More importantly however, the immunoblotting data are in agreement with our electrophysiology data showing that DCA restores I_K . The fact that Kv current is significantly diminished in the CH-PHT rats (Figure 1) is in agreement with the observed significant decrease in the expression of Kv2.1 (Figure 3), a 4-AP sensitive channel that contributes significantly contribute to the I_K in PASMCs.(7) The partial recovery of the lost 4-AP sensitivity in the PASMC of CH-DCA rats might indeed be due to the partial recovery of the Kv2.1 expression and function. The fact that the CH-DCA PASMCs appear to regain their responsiveness to acute hypoxia (although with a borderline statistical significance, $p=0.06$, Figure 5B) is likely due, in part, to the loss of the oxygen-sensitive channels, such as Kv1.5 and Kv2.1.

In addition to the chronic effects of DCA we show that that this drug can increase I_K acutely, much more rapidly and at lower doses than previously described (Figure 5). These effects might represent novel and previously unrecognized properties of DCA. Rozanski et al showed that cultured cardiomyocytes from infarcted rat hearts have increased I_K when incubated with 1.5mM DCA for 4 hours, compared to controls.(27) They showed that this was mimicked by pyruvate and inhibited by a PDH blocker and speculated that the effects of DCA were due to the metabolic actions of the drug and the activation of PDH.(27) We now show that DCA activates K^+ current in freshly isolated CH PASMC within 5 minutes and at the very low dose of 1 μ M. (Figure 5C). This dose is much lower than the dose required for the metabolic effects of DCA on PDH (mM range). Perhaps a different mechanism might be responsible for the electrophysiological effects of DCA. A major mechanism by which the function of K^+ channels is regulated is via phosphorylation by kinases. In many cases, phosphorylation activates K^+ channels (22). It is possible therefore that DCA modulates the function of kinases other than PDK at lower doses and thereby activates K^+ channels and hyperpolarizes PASMCs. More studies will be needed to address this intriguing possibility. Furthermore, DCA could affect the expression of K^+ channels via non-redox mechanisms. At least in neurons, membrane depolarization itself has been shown to cause Kv1.5 downregulation.(17) Assuming that such a mechanism is important in the pulmonary circulation,

DCA could affect the expression of Kv2.1 through its ability to activate I_K and thereby repolarize PASM C E_M .

Gene transfer in human PAs ex vivo

Although the adenoviral gene transfer studies are preliminary, they clearly demonstrate the ability to overexpress a functional Kv2.1 transgene in human PAs. Champion et al have previously demonstrated the feasibility of transferring genes for calcitonin gene related peptide (CGRP) and endothelial nitric oxide synthase (eNOS) to the mouse lung. (11, 12) Transfer of CGRP via airway delivery of adenoviral vector reduced the PHT and vascular remodeling seen in CH-PHT.(12). Champion et al also showed that gene expression after adenoviral gene transfer was sustained over a 21- to 28-day period (11). Twenty four hours after administration of adenovirus carrying eNOS, eNOS protein levels were increased, and there was a small reduction in mean PAP and PVR. The pulmonary vasodepressor responses

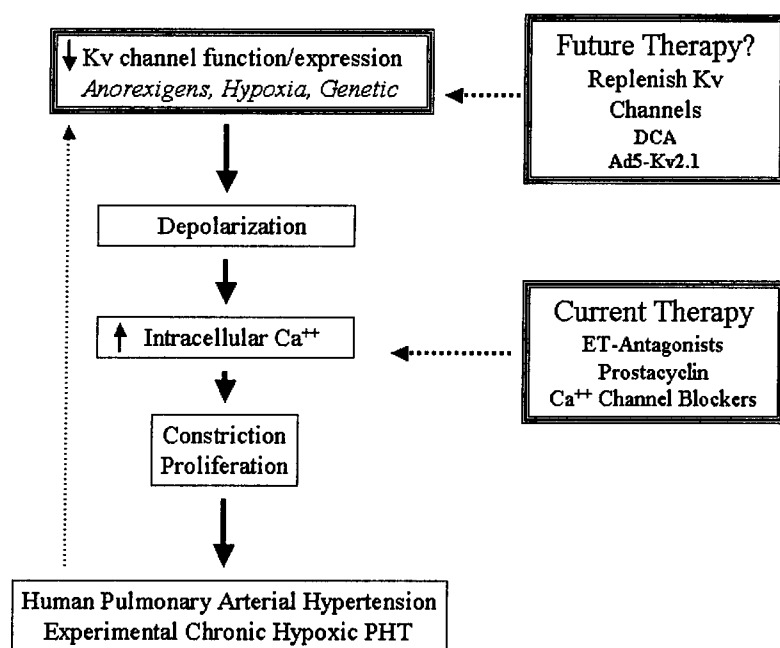


Figure 9. A schematic of our proposed mechanism by which loss of Kv channels could cause or exacerbate PHT. This diagram indicates the proposed steps in the pathogenesis of PHT and indicates the site of action of current versus future therapeutic modalities. The broken arrow on the left indicates the uncertainty as to whether loss of K^+ channels causes PHT or is the result of PHT.

to bradykinin and the type V cGMP-selective phosphodiesterase inhibitor zaprinast were enhanced, whereas systemic responses were not altered. Thus gene transfer via the airway is potentially an attractive therapeutic strategy.

The current work, although preliminary, extends this by using a vector that can be detected noninvasively due to the inclusion of GFP (Figure 6). GFP indicates the location and amount of gene transferred and because it is in the same vector as the gene of interest can indicate the amount of transgene delivered. Using Chinese Hamster Ovary cells (CHO) we showed that the greater the dose of Ad5-GFP-Kv2.1, the greater the fluorescence.

SIGNIFICANCE

This is the first demonstration of the successful transfer of a Kv channel to a human PA, to our knowledge. Although the benefits of K⁺ channel supplementation remains untested, it is feasible to restore reactivity to Kv channel blockers in PA rings from humans with PAH. This research may have relevance to patients with cor pulmonale because although CH-PHT is reversible on return to normoxia, correction of hypoxia is often impossible in patients with chronic lung disease. Future studies will need to include careful time course studies and will require confirmation of the effects of gene transfer and protein expression on cellular electrophysiology.

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Chapter 27

Chronic mountain sickness

A view from the crow's nest

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Abstract: Chronic mountain sickness (CMS) is a poorly understood syndrome, characterized by hypoxemia and polycythemia and occurring in persons residing at high altitude. To better characterize the disorder, we have reviewed measurements in more than 750 men and 200 women living at altitude as published and as submitted by colleagues. In men, blood hemoglobin concentration (Hb) and arterial oxygen saturation (SaO₂) related to altitude ($r=0.72$). There was greater variability in both SaO₂ and hemoglobin above than below 3000 m, largely due to inter-individual variations in effective ventilation. For the entire cohort, a linear relationship ($r=0.72$) of an index of hematopoietic response (Hb) to an index of stimulus (SaO₂) was independent of age, altitude, duration of altitude residence greater than one year, ethnic origin, geographic location, presence or absence of CMS and nearly independent of gender. A potentially important and usually unrecognized variation in the hypoxic stimulus was desaturation during sleep. Contributions to variation in response include ingested toxins, such as cobalt, and nutritional deficiencies, including iron. Pulmonary hypertension was related to chronic hypoxia, with an uncertain contribution from polycythemia. In CMS there were profound hypoxemia at night, decrease in cerebral blood flow, and loss of cerebral blood flow regulation, possibly causing the cerebral symptoms. We speculate that the relationship of Hb to SaO₂ is more useful than of hemoglobin to altitude, that hypoventilation awake and asleep are the primary causes accentuating altitude-hypoxia, and that the brain is the primary target organ in the disorder.

Key words: altitude, polycythemia, erythropoietin, pulmonary hypertension, sleep

INTRODUCTION

"Chronic mountain sickness (Monge's disease) is the occurrence of symptomatic, excessive polycythemia in long-term residents of high altitudes. Hypoxia is the stimulus to erythropoiesis; symptoms result from a severe expansion of blood volume and a consequent burden on the circulatory system. (38)" So begins the classic book on the subject by Robert M. Winslow and Carlos Monge C. In addition, these authors published an excellent historical account of the origins of understanding chronic mountain sickness, or CMS as we will refer to it. In the first description of the disorder, Carlos Monge M. in 1925 recognized the central role of hypoxia, as indicated by the subtitle for the paper, "Erythremic Syndrome of Altitude" (22).

At the end of their book, following an exhaustive analysis, Winslow and Monge published a schema summarizing their concepts for the etiology of CMS. Interestingly, while their schema highlights hypoventilation during wakefulness and sleep, which augments hypoxia at altitude, and points to hypoxic stimulation of erythropoietin and polycythemia, it does not indicate the nature and extent of linkage of hypoxemia and polycythemia to the CMS syndrome. Probably this was wise and perhaps deliberate, for this is the difficulty which we face today. Our hypothesis, building on that of the International Working Group for Chronic Mountain Sickness (6,13,26), is that the target organs in CMS are the lung and, in particular, the brain. Of the two, we will suggest that the brain is the more susceptible. Using a literature review, we will try to approach our hypothesis by examining questions relating to variability in the hypoxic stimulus, and the hematopoietic response, as well as the likely clinical consequences.

METHODS

To approach the questions, we have reviewed the individual reported values relating to altitude, hypoxia, erythropoietin, and polycythemia, of persons living at altitude, with the intent of displaying and examining the variation among individuals. Because the hypoxic stimulus and the hematopoietic response was variable among reported residents at high altitude, we have first tried to determine the magnitude of the variations. Because the commonly reported index of oxygenation was percent arterial oxygen saturation (SaO_2 %), and the most commonly reported index of polycythemia was hemoglobin (Hb gm/100 ml), our analysis utilized these variables. Where hematocrit, and not hemoglobin, was reported we assumed hemoglobin concentration in gm/100 ml was one third the percent hematocrit. For sub-sections A and B in the following section, we used

individual measurements in 741 men and 213 women as reported from the literature (3,5,7,8,9,10,20,24,25,27,29,33,35,36,37) or as obtained as personal communications from various authors (see Acknowledgements). From examination of available data, we entered for analysis hemoglobin, SaO₂, altitude, geographical location. We also noted as CMS those individuals considered to have either chronic mountain polycythemia or CMS. We do not claim to have reviewed all published literature, nor can we claim that the data are a random aliquot of any population.

For examination of the pulmonary circulation, sub-section C, below, we used individual measurements as reported for 145 persons studied by heart catheterization at various altitudes (5,9,10,24,25,27). The analysis of measurements during sleep, sub-section D, below, were from the study of Sun et al. (35).

RESULTS AND DISCUSSION

A. Altitude, Hemoglobin, and Arterial Oxygen Saturation

The first question was how an indicator of the stimulus, SaO₂, and an indicator of the response, hemoglobin, varied with altitude. Individual values for men showed, as expected, that with increasing altitude ranging from sea level to nearly 6000 m, arterial saturation fell ($r=-0.72$, $p<0.001$) and blood hemoglobin rose ($r=0.72$, $p<0.001$). But individual variation in both SaO₂, and hemoglobin with altitude was greater for altitudes above, than for those below, 3000 m, Figure 1A,B. The implication was that factors relating to residence at the higher altitudes increased the inter-individual variation in both stimulus and response.

Those factors were, in large measure, breathing and the shape of the hemoglobin – O₂ dissociation curve. At low altitude, for example 1600 m, breathing had little or no influence on SaO₂, Figure 2A, or blood hemoglobin concentration, Figure 2B, because there was a flat relationship of SaO₂ or of hemoglobin to PCO₂. At 3100 m, the effect of breathing on SaO₂ and hemoglobin concentration began to be apparent (legend, Figure 2). At 4300 m altitude and even more at 5950 m, breathing had a major influence on hemoglobin concentration, where those who breathed more (lower PCO₂ values) had higher SaO₂ and lower hemoglobin than those persons who breathed less (higher PCO₂ values), Figure 2A,B. For a given altitude above 300m m, breathing was a key determinant of hemoglobin concentration. Furthermore, with increasing altitude, the progressively increasing absolute values of slopes of the SaO₂ - PCO₂ and the hemoglobin - PCO₂ relationships indicated greater influence of breathing on hemoglobin – a finding which reflected the shape of the hemoglobin – O₂ dissociation curve.

At low altitude, where persons function on the flat portion of the dissociation curve, the usual inter-individual variation in breathing would have little effect on SaO_2 , and hence on hemoglobin. But with increasing high altitude as persons function on the steeper part of the curve, breathing has an increasingly important effect.

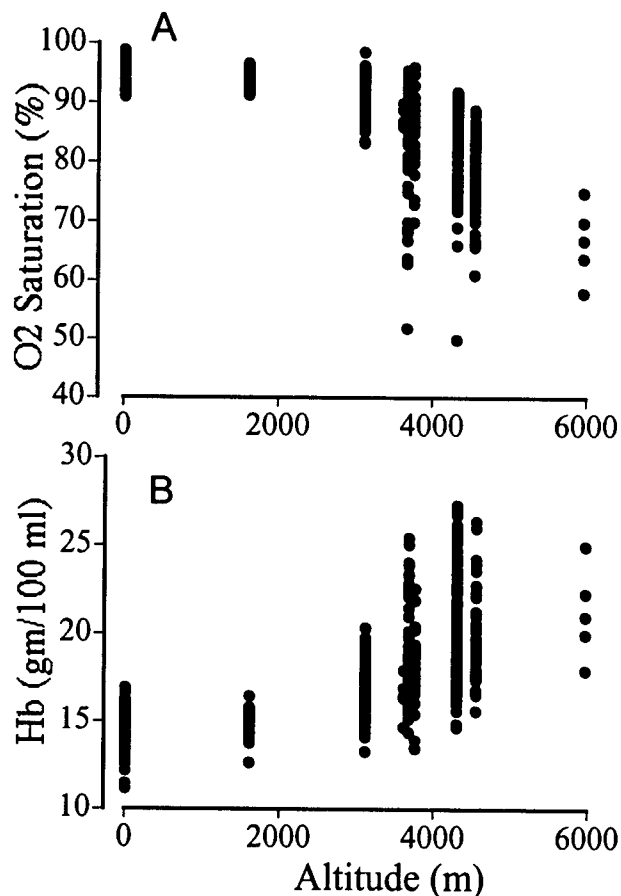


Figure 1. Relationship of altitude to arterial oxygen saturation (Panel A, top) and to Hemoglobin (Panel B, bottom). Shown are data points for 727 individual men as collected from published literature, and from unpublished measurements obtained by personal communication. Note the large variability of the measurements at altitudes between 3000 and 5000 m.

Because variations in breathing, which alter SaO_2 at altitude, are determinants of hemoglobin, we should for investigative purposes relate hemoglobin level (a measure of the hematopoietic response), to SaO_2 (a measure of the stimulus), rather than to a third variable, altitude. Not only should a stimulus-response relationship provide an approach to increase our

understanding, but also we hoped the inter-individual variation in the hemoglobin - SaO_2 , relationship would be less than when each of the two variables was related to altitude. In hoping for a decrease in inter-individual variation, we were disappointed, for the correlation coefficient for the hemoglobin- SaO_2 relationship, Figure 3A, was practically identical to those relating hemoglobin to altitude or saturation to altitude. However, for the entire cohort, a clear linear relationship of hemoglobin to SaO_2 was obtained, Figure 3A.

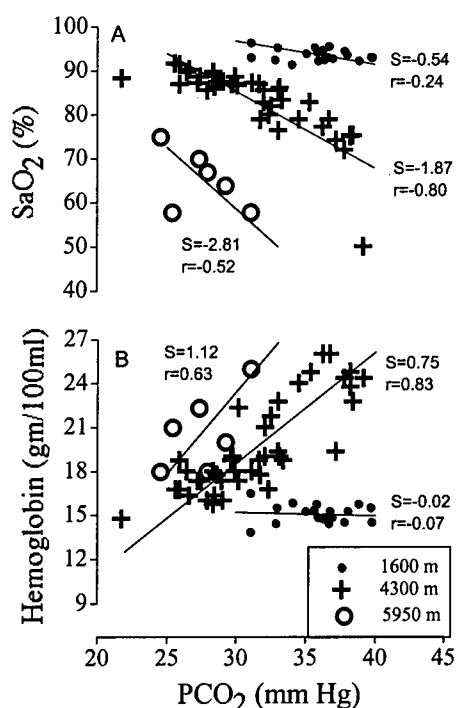


Figure 2. Panel A, top. Relationship of arterial oxygen saturation (SaO_2) to arterial or alveolar carbon dioxide pressure (PCO_2) for the three altitudes, 1600 m (37), 4300 m (34), and 5950 m (29) as shown. Slopes of the relationships, S, as the reduced major axis and the correlation coefficient, r, are shown for each altitude. Not shown are slopes of -0.70 for 3100 m (37) and -0.97 for 4540 m (27). Panel B, bottom. Relationship of hemoglobin to arterial or alveolar carbon dioxide pressure (PCO_2) for the three altitudes, 1600 m (37), 4300 m (34), and 5950 m (29) as shown. Slopes of the relationships, S, as the reduced major axis and the correlation coefficient, r, are shown for each altitude. Not shown are slopes of 0.45 for 3100 m (37) and 0.52 for 4540 m (27).

Of interest and a surprise to us, was that in the combined data in Figure 3A, many dissimilar groups joined to form a single linear relationship. That is, the relationship shown in Figure 3A appeared independent of age for the

members of the cohort. Also, altitude of residence, duration of residence beyond one year, geographic location, ethnic origin, employment, or whether the men were or were not diagnosed as having chronic mountain sickness - none of these altered the hemoglobin- SaO_2 relationship. For the combined data assuming a linear relationship, the correlation coefficient ($r = -0.72$) and an r^2 of 0.52 suggested that approximately 52% of the variation in hemoglobin values could be accounted for by the arterial oxygenation alone. We had expected, for example, that for a given SaO_2 , older men would have higher hemoglobin than younger men, because the incidence of CMS increases with age (3,14,16,19,21,38). Perhaps the large variation within our cohort had obscured an independent effect of age. However, we found the relationship of hemoglobin to SaO_2 in men less than 30 years was identical to that in men older than 40. In our cohort, SaO_2 fell with advancing age at 4300 and 4540 m ($p < 0.05$), but not at 3100m, or at sea level, and hemoglobin rose with advancing age at 4300 and 4540 m ($p < 0.05$), but not at the lower altitudes. Such findings did not deny that age was a factor in polycythemia at altitude, but rather the findings implied that a decreasing SaO_2 with advancing age was likely responsible for an increasing

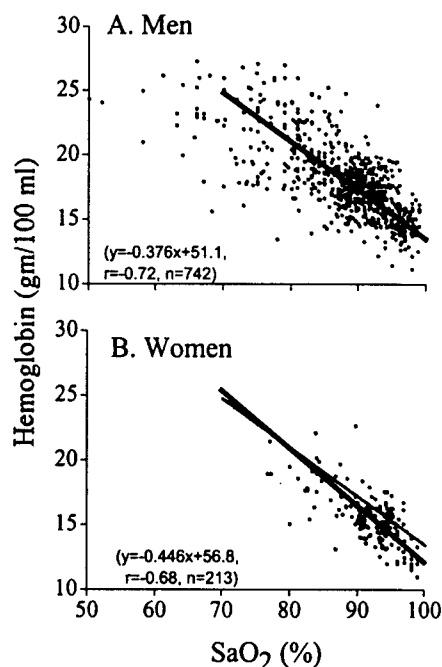


Figure 3. Relationship of arterial oxygen saturation (SaO_2) to hemoglobin in men (Panel A, top) and women (Panel B, bottom) men as collected from published literature, and from unpublished measurements obtained by personal communication. The heavy line of best fit and the regression equation, correlation coefficient, and number of individuals are shown. The line of best fit for men in Panel A is reproduced as a broken line in Panel B, and suggests that the difference in hemoglobin between men and women diminishes with reduced SaO_2 .

hemoglobin concentration. If so, we concluded that the hemoglobin would be higher in the older men simply because they were more hypoxic than the younger men (38).

The findings also indicated an absence of ethnic effect. We had expected higher hemoglobin values in relation to SaO_2 in the Han than the Tibetan residents of Lhasa, for Han residents are much more susceptible to CMS than the Tibetan natives (23). However, the analysis found that the various geographic or ethnic groups all showed the same relationship of hemoglobin to SaO_2 . Whether subjects were Han or Tibetan or Peruvian, or highlanders or lowlanders, or living in Leadville, CO, all showed the same relationship of hemoglobin to SaO_2 . Furthermore, we had certainly expected CMS patients to have higher hemoglobin values (in relation to SaO_2) than healthy altitude residents, but this expectation, also, was not realized. The data in men from the three continents showed nearly complete overlap and there was considerable overlap in persons considered to have CMS and those with no such diagnosis.

Importantly, menstruating women have lower hemoglobin levels than men at sea level, where, because of the shape of the Hb- O_2 dissociation curve, SaO_2 values are nearly independent of gender. And so we expected women, particularly those in the childbearing age, would not have the same hemoglobin- SaO_2 relationship as men. Progesterone is a known ventilatory stimulant and young women have lower hemoglobin values than men at all altitudes which have been studied (16,17,23,40,41). While fewer data were found for women than men, the hemoglobin - SaO_2 relationships were remarkably similar for men and women, particularly in women with lower values of SaO_2 , Figure 3B. The implication for both men and women is that hemoglobin concentration relates rather well to SaO_2 .

If so, what is meant by the term "excessive erythrocytosis"? As properly employed, the term indicates excessive concentrations of red cells or hemoglobin or levels of packed cell volume for a given altitude, not for a given SaO_2 , a distinction which has long been made by Winslow and Monge (38). Unless properly defined the term has potential for inducing confusion. Furthermore, while it may be clinically useful for diagnostic purposes to establish hemotologic norms for a given altitude population, for better understanding of pathogenesis, and even for treatment, it is important to emphasize that much of the variation in hemoglobin at altitude reflects variation in oxygenation. Because for a given patient, the inquiry must be directed at whether the SaO_2 is low, and if so, why, we suggest wider use of hemoglobin - SaO_2 relationships. We are not aware that these hemoglobin - SaO_2 relationships have been displayed before for such large cohorts of individuals. As suggested below, the mean lines describing these relationships might be useful for clinical studies of the normal and abnormal responses to altitude.

B. Factors Varying the Hematopoietic Stimulus and Response

Even if we employ the hemoglobin - SaO_2 relationship as proposed, how can we account for the 50% of the variation in hemoglobin, which is not explained by SaO_2 ? For our cohort, we examined the variation in hemoglobin as hypoxemia increased in severity. We saw that the highest hemoglobin levels did not occur in persons with the lowest saturations. The single highest hemoglobin was 27.3 gm/100 ml, and there were five men with hemoglobin values of 27 g/100 ml or more. However, their SaO_2 values ranged from 66% to 79%; yet there were other men with SaO_2 values below 60% with hemoglobin values well below 25 gm/100 ml, Figure 3A. Presumably, there must be an upper level for blood hemoglobin concentration and hematocrit if the blood is to remain sufficiently fluid to circulate. Possibly, hemoglobin levels above 27 gm/100 ml are soon fatal, so that when SaO_2 is less than 70% only those persons with a lesser hematopoietic response survive. Monge C. has pointed out that for saturations less than 70 to 75%, the hematopoietic response cannot compensate for the low SaO_2 (21). We do not know whether, for high hemoglobin or severe hypoxemia, there is some inhibitory influence on hematopoiesis. Whatever the reason, the limit to the hematopoietic response to hypoxia is set at an SaO_2 of 65 to 70 % .

Such limitation was more clearly seen by examination of hemoglobin levels for classes of SaO_2 . For the entire cohort of men, as the SaO_2 became lower the hemoglobin approached a plateau at an SaO_2 value of about 70%, Figure 4A. Also, the maximal standard deviation in hemoglobin occurred near the same SaO_2 level of ~70%. The coefficient of variation, which is the standard deviation divided by the mean, confirmed that variation in hemoglobin increased as SaO_2 fell to values approaching 70%, and then declined when SaO_2 decreased below that value, Figure 4B. In part as noted above, the higher hemoglobin variability with increasing hypoxia probably reflected the shape of the Hb- O_2 dissociation curve, where small decrements in PO_2 cause large decrements in SaO_2 . If so for example, inter-individual differences in ventilation would have a much larger effect on SaO_2 (and hence on hemoglobin) at altitude than at sea level.

In addition, a large part of the variation in the hemoglobin - SaO_2 relationship for the reported data, may reflect sampling error in estimating the hypoxic stimulus. Making a nearly instantaneous measurement and relating it causally to a chronic response, is fraught with potential for error. From this point of view, the saturation measurements were crude, even if we discount that we had no good tissue PO_2 measurement within the renal perivascular fibroblasts, presumably the site where hypoxia is sensed. Nor did we know the round the clock average of the hypoxic stimulus, which

related to the hematopoietic response. Rather, the reported SaO_2 values were single, random, daytime measurements. At high altitude, where subjects function on the steep part of the Hb-O₂ dissociation curve, the body's oxygenation will vary markedly within and between subjects during the waking hours.

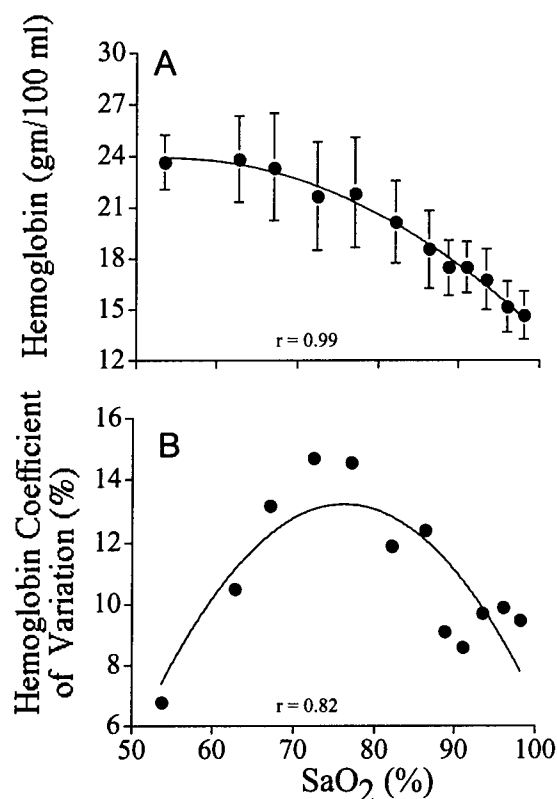


Figure 4. Panel A, top: Relationship of average hemoglobin values to arterial oxygen saturation (SaO_2) classes where the classes are in increments of 5%. Shown for a total cohort of 741 men are mean values with one standard deviation above and below the mean.

An line of best fit has been drawn through the data. Panel B, bottom: Relationship of variability in hemoglobin measurements to arterial oxygen saturation (SaO_2) classes where the classes are in increments of 5%. The index of variability is the coefficient of variation (a ratio in % of one standard deviation divided by the mean). Note that at SaO_2 values below 70 to 75% the hemoglobin concentration reaches a plateau and the variability decreases.

Sleep-induced variations of the hypoxic stimulus within and between subjects at altitude was probably an important contributor to variable hematopoiesis. Even at sea level when normal persons go to sleep,

ventilation decreases and there are periods of irregular breathing. Both the decreased ventilation and the irregular breathing become augmented during sleep at altitude (12,35). At altitude, largely as a result of the alveolar hypoxia and the shape of the dissociation curve, even normal people show a greater decrease and greater variability in nocturnal SaO_2 than at sea level. Persons with disordered nighttime breathing living at altitude, can experience profound hypoxemia at night. If nocturnal hypoxemia promotes erythropoiesis, as has been suggested (12), then measurement of daytime SaO_2 will underestimate the hypoxic stimulation to red cell formation.

During sleep at 3658 m, patients with CMS spent two thirds of their night with SaO_2 values less than 70% (35), Figure 5. For a quarter of their time asleep, SaO_2 was less than 50%! For healthy young people and even for healthy persons the same age or older, SaO_2 during sleep rarely fell to or below 70%. Evidence that nocturnal hypoxia at altitude impacted hematopoiesis, was obtained by Kryger et al. (12), who showed that improving nocturnal oxygenation decreased hemoglobin levels and ameliorated the symptoms of CMS. Thus, for persons with disordered nocturnal breathing at high altitude, the SaO_2 during the waking hours may be an inadequate estimate of the hypoxic stimulus for erythropoiesis.

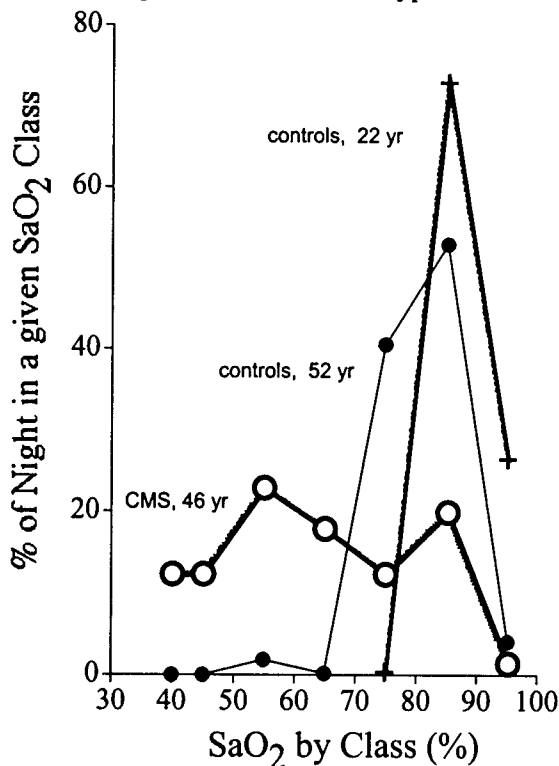


Figure 5. Nocturnal arterial oxygen saturations during sleep in Lhasa, Tibet at 3658 m altitude. Shown along the abscissa are arterial oxygen saturations (SaO_2) by classes of 10%, and along the ordinate are the percentages of time during the night spent in a given SaO_2 class. Note that in 22 year old controls values for SaO_2 did not fall below 75%, and in 52 year old controls, only a negligible part of the night was spent below 70% SaO_2 . However, for patients with chronic mountain sickness (CMS), more than half the night was spent at SaO_2 values below 70%. (Adapted from reference 35).

However, we still know very little about how sleep impacts erythropoietin production at high altitude. For example, in the cohort presented, we would have expected subjects with severe hypoxemia to have excessive polycythemia relative to their daytime SaO_2 , but that was not the finding. Furthermore at sea level, nocturnal hypoventilation and obstructive sleep apnea, with episodes of very low SaO_2 values, does not result in polycythemia. Further research is needed to establish whether or not sleep inhibits the production of erythropoietin.

In addition to variations in the hypoxic stimulus, there are numerous variables affecting the hematopoietic response. Cobalt is a powerful stimulus for erythropoiesis, which mimics the effect of hypoxia. Furthermore, cobalt can find its way into the drinking water of mining communities. Jefferson et al. in this symposium (11) report the presence of Cobalt in the blood of some persons living in the mining community of Cerro de Pasco at 4300 m in the Peruvian Andes. The cobalt level related positively to the hemoglobin concentration, and the hemoglobin levels which they found, though correlated with the degree of hypoxemia, were consistently above the mean value we have reported for the whole cohort studied, (11 and Johnson and Schoene, personal communication). Cobalt ingestion is a potentially important factor increasing variation in hemoglobin- SaO_2 relationship in mining communities

Though erythropoietin is generally appropriate for the hypoxic stimulus at altitude (2,15), it is a potential source of variation. One variable which reduces the hematopoietic response to hypoxia even in the presence of elevated erythropoietin, is low availability of substrate for hemoglobin formation, particularly decreased iron stores. In a large cohort of men and women examined in Leadville, CO at 3100 m altitude, subjects with low levels of blood ferritin ($<20 \mu\text{g/liter}$), an index of iron stores, had high blood levels of erythropoietin (Asmus, personal communication), the primary hormone stimulating new red cell formation. In some of these subjects, for example menstruating women or persons undergoing frequent phlebotomy, the increase in erythropoietin was an inadequate compensation for a markedly reduced iron availability, and the hemoglobin values were below those expected for the level of SaO_2 .

From the above it seems that the relationship of hemoglobin to SaO_2 is increasingly variable with increasing hypoxia, at least until some limiting factor(s) come into play at hemoglobin concentrations at approximately 25 gm/100 ml. Causes of the variability at altitude involve our inability to accurately identify both the stimulus and the mechanisms of response. Prominent among the former are SaO_2 measurements which do not reflect the true stimulus, particularly when they ignore oxygenation during sleep. Prominent among the latter are the possibility of cobalt ingestion in mining communities and nutritional deficiencies, such as in iron which limit the

hematopoietic response. While the present report has drawn attention to the importance of relating hemoglobin to a measure of oxygenation, in order to establish better the normal relationship, our analysis is crude. In future work, factors which should not be ignored include a better assessment of the hypoxic stimulus, the hormonal environment of the subject, and total red cell mass, to name a few.

C. The Pulmonary Circulation

Both hypoxia and a polycythemia-related increase in viscosity are considered to increase pulmonary vascular resistance and to increase pulmonary artery pressure. For example, in animal experiments the combination of polycythemia plus acute hypoxia increased pulmonary vascular resistance more than either alone (18). But separating the effect of chronic hypoxia from viscosity is a daunting task in humans. One approach is to consider the relation of pulmonary vascular resistance to SaO_2 . In examining reports of nearly 150 persons having heart catheterization at various altitudes, there was a strong relation of pulmonary resistance to hemoglobin ($r=0.73$) and to SaO_2 ($r=-0.72$), but there was no such relationship of the systemic resistance to these variables ($p=\text{ns}$), Figure 6. Even though hypoxia induces pulmonary vasoconstriction and systemic vasodilation, if extreme polycythemia-related viscosity were important, we had expected to see some systemic hypertensive effect of a high hemoglobin. But no such effect was observed in this cohort.

Of interest, pulmonary vascular resistance was found to be normal in polycythemia vera where the hemoglobin was elevated ($>19 \text{ gm}/100 \text{ ml}$), but the lung was normal and there was no hypoxemia, Figure 7A (31). One might expect the polycythemia to increase viscosity and thus the resistance to blood flow through the lungs, but this was not found. The explanation given was that an expanded central blood volume dilated the lung microcirculation and counteracted any effect of high viscosity (31). While an increased pulmonary blood volume might be expected to dilate the thin walled, normal lung arterioles, an increased blood volume might not be expected to dilate arterioles with thickened, muscular walls. Yet, in patients with bronchitis-induced chronic hypoxemia and polycythemia and who had chronically elevated pulmonary vascular resistance, phlebotomy to lower hemoglobin resulted in only a small decrease in resistance (32). In the analysis of his own data in high altitude residents and that of Penalosa (25), Hultgren (10) concluded that high hematocrit had no influence on pulmonary arterial pressure or resistance. Taken together, these findings suggested that chronic polycythemia did not have a great effect in increasing resistance to blood flow through the hypoxic lung even when the lung vascular bed was abnormal.

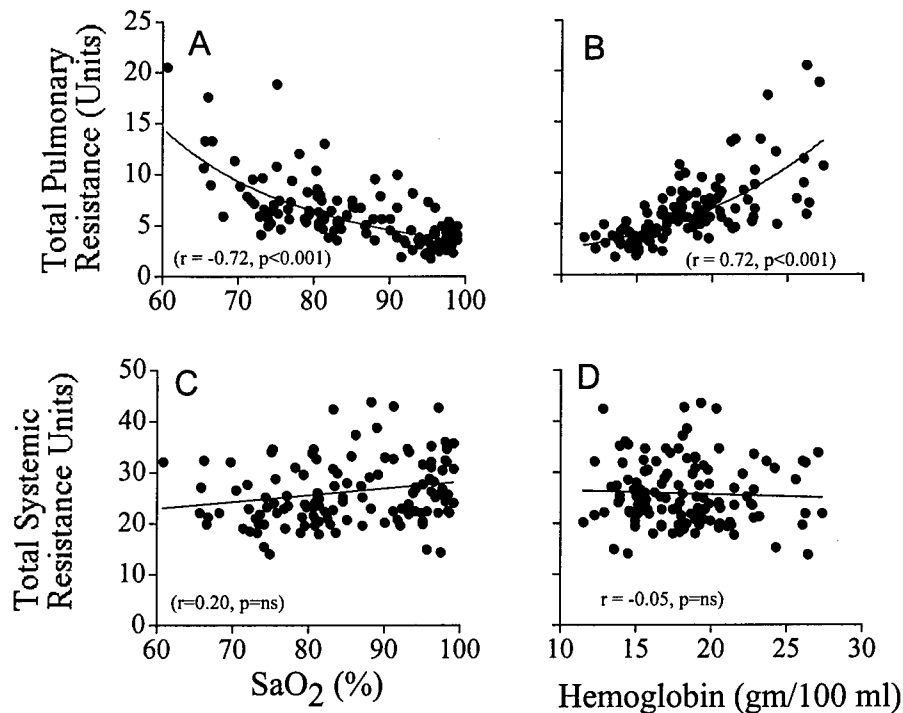


Figure 6. Relationships of total pulmonary vascular resistance to arterial oxygen saturation (SaO_2), Panel A, and hemoglobin, Panel B, as measured during heart catheterization in 145 men at various altitudes. Significant increments in pulmonary vascular resistance were seen with increasing hypoxia and with increasing hemoglobin. No such increases were noted in the total systemic vascular resistance, Panels C and D.

To approach this issue for the high altitude native, we compared the relationship of pulmonary vascular resistance to hemoglobin for higher and lower concentrations (i.e., <19 versus >20 gm/100 ml) of hemoglobin in the population of persons having heart catheterization at various altitudes. The results, Figure 7B, suggested that the cohort with the higher hemoglobin shifted the relationship (of hemoglobin to pulmonary vascular resistance) to the right. That is, for a given hemoglobin level, resistances were lower in the high than in the low hemoglobin cohort. It was a surprising result, for if true, the implication was that polycythemia acted to lower rather than raise the resistance to blood flow through the lung. While the above analyses suggest that hypoxemia increased pulmonary vascular resistance at high altitude more than polycythemia, the evidences were all indirect.

In support of the concept that high hemoglobin contributed significantly to resistance to blood flow through the lung at high altitude was the report by Winslow et al. (39), which directly studied the question. A carefully controlled hemodilution study, which lowered hemoglobin from 22.4 to 16 gm/100 ml, in one subject with chronic altitude-induced polycythemia led to

a large reduction in pulmonary vascular resistance as measured within two hours of the procedure, Figure 7A (39). Even though the SaO_2 rose from 80 to 84 %, one wonders if this, of itself, could account for the large fall in resistance. After four days, repeat catheterization in this subject showed even further reduction in pulmonary vascular resistance. We are not aware of other studies, which have measured the effect of lowering hemoglobin concentration on pulmonary hemodynamics in normal high altitude residents or those with chronic mountain sickness, but the findings in this one subject support the importance of high hemoglobin in the pulmonary hypertension of altitude.

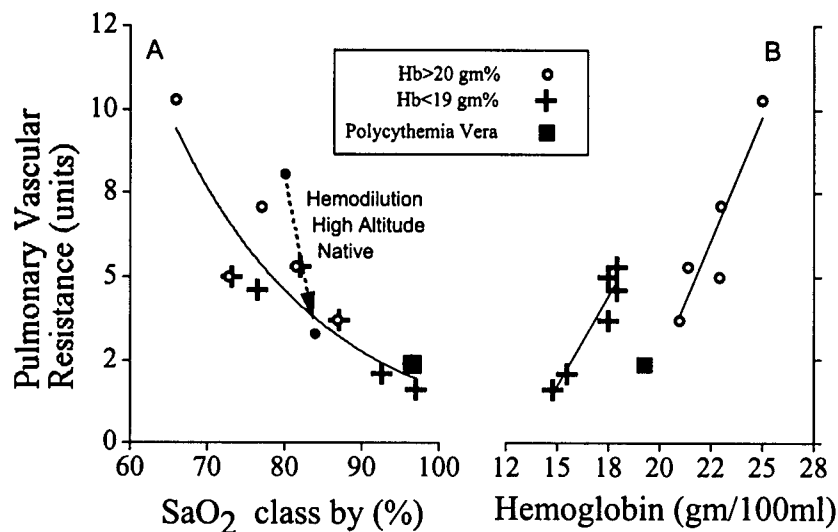


Figure 7. Panel A, left. Relationship of pulmonary vascular resistance to arterial oxygen saturation (by SaO_2 class of 5%) for 33 men with hemoglobin values greater than 20 gm/100 ml (unfilled circles) and 68 men with hemoglobin values less than 19 gm/100 ml. For SaO_2 classes of 72, 82 and 87 % the resistances were not different between groups with higher versus lower hemoglobin values. Mean measurement from 27 persons with polycythemia vera (filled square), indicate no pulmonary hypertension at sea level (31). Filled circles connected by the broken arrow indicate reduction in hemoglobin from 22.4 to 16 gm/100 ml by hemodilution performed over two hours in one high altitude native (39). Panel B, right. Relation of pulmonary vascular resistance to hemoglobin levels for the arterial oxygen classes shown in Panel A. Lines of best fit are shown. Note that the open circles indicating higher hemoglobin levels do not lie on the same line with the crosses, which indicate lower hemoglobin. The 27 polycythemia patients, with a mean hemoglobin of 19.2 gm/100 ml (31) are associated with the higher hemoglobin in this plot.

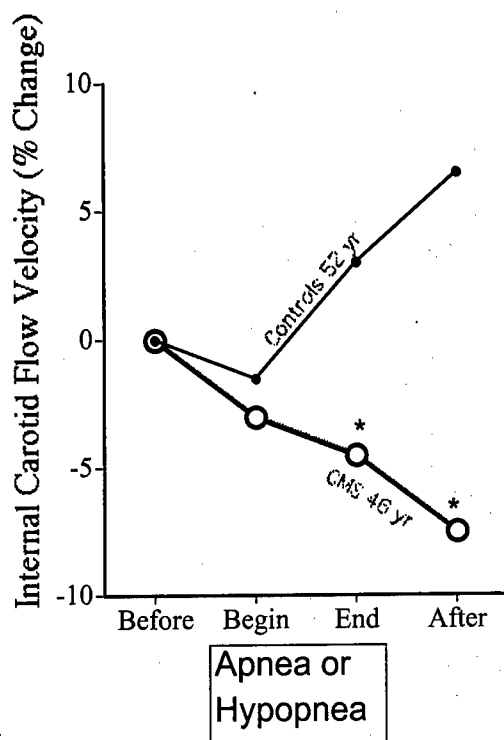


Figure 8. Relationship of internal carotid Doppler flow velocity to breathing pattern during sleep in men in Lhasa, Tibet, at 3658 m. Shown are changes in flow velocity in association with episodes of apnea or hypopnea lasting at least ten seconds. Note that in eight healthy men age 52 years (filled circles and unbroken lines), episodes of apnea or hypopnea were accompanied by increased internal carotid flow velocity, whereas in the eight men with chronic mountain sickness (CMS) flow velocity decreased (indicated by asterisks). (Redrawn from reference 35).

Thus, when one asks how high hemoglobin, per se, affects the lung circulation at high altitude, the answers are not clear. It is our speculation, that the chronic hypoxia of high altitude is likely a more important influence than the level of hemoglobin in raising pulmonary vascular resistance, but we are not sure. At present we cannot exclude the possibility that severe polycythemia (when in the presence of chronic, severe hypoxemia) contributes significantly to the resistance of blood flow through the lung. Even so, while some degree of pulmonary hypertension is reported as a consistent finding in patients with high altitude polycythemia (4,7,9,27,28), right heart failure is likely to be a late and possibly inconsistent manifestation of CMS (4,28). Clearly further research is needed in this area.

D. The Brain as the Primary Target in Chronic Mountain Sickness?

With sub-acute hypoxia, when normal volunteers in Operation Everest II were decompressed over nearly six weeks to the equivalent of the summit of Mt. Everest, the central nervous system, particularly the brain, was the primary target organ. Considering the well known susceptibility of the brain to hypoxia and for the following additional reasons, we wonder if the brain is also the most likely primary target organ in CMS: A) In CMS, the most common symptoms include physical and mental fatigue, feelings of sadness, headache, tinnitus, sleep disturbance, dizziness and anorexia (1,4,6,14,17,38) and they all point to malfunction of the brain. B) Mental confusion is a commonly cited as sign and symptom in CMS patients, as illustrated by the dramatic initial description of the disorder (22). C) Severe polycythemia is thought to impair normal microcirculatory perfusion. D) Brain blood flow is decreased in CMS (35). E) Sleep disturbances are increased as indicated by the increased number and duration of periods of apnea and hypopnea, and such periods are associated with profound hypoxemia (12,35). G) The profound hypoxemia associated with extreme polycythemia is associated with a loss of regulation of the cerebral circulation with regard both to low oxygen and high carbon dioxide (35).

Medical scientists from around the globe have struggled with a definition of the CMS syndrome (6,13). Symptoms are subtle, and their appearance may be either intermittent or so gradual that the patient may not be aware of their presence. People at risk may deny the presence of symptoms in order to preserve their occupations. Furthermore, the individual tolerance of hypoxemia or polycythemia is so great that the measurements of random SaO₂ or hemoglobin do not provide very precise indexes of chronic mountain sickness. How, then, should we proceed?

One approach would be for investigators to develop signs and symptoms which more precisely than heretofore, relate to the central nervous system, particularly the brain. Simple tests of mental function involving memory, ability to maintain concentration, capacity for muscular coordination, facility in performing simple calculations – all might be of use. Such tests might be performed in hypoxia with subjects breathing ambient air at altitude, and during oxygenation to monitor whether or not there was improvement. Non-invasive measurements of cerebral blood flow regulation in response to changes in O₂ and CO₂ tension would also provide important information which would increase understanding of the disorder and might be helpful clinically in its early detection. Recently a world-wide working group of investigators has come together to study CMS (13), and we hope that this view from the crow's nest will be useful in its deliberations.

ACKNOWLEDGMENTS

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Chapter 28

International Working Group For Chronic Mountain Sickness

Jasper Park Lodge, Canada 13 March 2001 – 1:00 to 2:00 PM.

Present: Ingrid Asmussen (USA), Luciano Bernardi (Italy), Peter Bärtsch (Germany), Tom Hornbein (USA), Fabiola Leon-Velarde (Peru, Chair), Marco Magiorrini (Germany), Jim Milledge (UK), Jean-Paul Richalet (France), Jack Reeves (USA), Rob Roach (USA), Brownie Schoene (USA), Steve Wood (USA), Enrique Vargas (Bolivia), Ken Zafren (USA).

MEETING MINUTES

1. Chairwoman Leon-Velarde opened the meeting and reviewed the working group's activities since the last meeting in Matsumoto, Japan.

A letter from Peru and Kirghizstan was sent to the World Health Organization (WHO) alerting them to the magnitude and severity of chronic mountain sickness and recommending that consideration be given to this disorder as an international health problem. While the WHO expressed interest, there has as yet been no action. The international database which the Working Group intends still needs to be established.
2. There was considerable discussion on chronic mountain sickness pointing to the many needs, among which were:
 - a. Epidemiological studies relating to severity and magnitude with regard to all ages
 - b. Physical and mental development in children
 - c. Actuarial studies relating to hemoglobin concentration (analogous to those which established normal values for blood pressure)

- d. For those who do not survive, the causes of death
 - e. Longitudinal studies within individuals in populations
 - f. International cooperation both in planning and conduct of studies related to the problem
 - g. More data collection in order to begin to establish definitions and guidelines
 - h. Risk of CMS in native born versus newcomers to altitude
3. In view of the poor understanding of chronic mountain sickness, the working group considers to begin by examining normal values for hemoglobin and hematocrit. The one could begin to establish guidelines for high altitude excessive erythrocytosis (HAEE). A subsequent step would then be to examine how HAEE developed into a clinical syndrome of chronic mountain sickness. With this overall strategy the group planned first to collect simple data in healthy individuals for all available altitudes, with respect to age and gender, for hemoglobin, hematocrit, and arterial oxygen saturation. Jack Reeves, Rob Roach and Steve Wood agree to begin to organize such data. Additionally, the data will be available for all contributors and other interested parties. The consensus was that such data in normal subjects would be a foundation for considering abnormalities.
 4. It was anticipated that some initial data from this effort would be available for presentation at the International Society for Mountain Medicine (ISMM) meeting to be held in Barcelona (Spain) in April of 2002. Regarding ISMM meeting, the recommendation was that there be an emphasis session for CMS. Investigators were urged to present their work in this area at that meeting. Marco Magiorrini will work with the ISMM meeting chairperson to help develop a program on CMS/Excessive Erythrocytosis. The possibility was raised that representatives or WHO might be present at the session.
 5. Chairperson Fabiola Leon-Velarde was re-elected to the Chair of the Working Group.

Respectfully submitted, March 2001 by John T. Reeves

Chapter 29

Late and Amended Abstracts

EFFECTS OF THE INTERVAL HYPOXIC THERAPY IN NON-INSULIN-DEPENDENT DIABETES MELLITUS (NIDDM). DOUBLE BLIND PLACEBO CONTROLLED STUDY. Ehrenburg I.V., Starkova N.T*, Davydov A.L.*, Tkatchouk E.N. *Moscow Medical University of Stomatology and Clinical Research Laboratory "Hypoxia Medical Academy", Moscow, Russia

The prevention of vascular complications in diabetes mellitus patients is important. It is known that glycemia NIDDM compensation does not prevent their development. One of the causes of the diabetic vascular complications and neuropathy progress is circular and tissue hypoxia. The IHT was used to manage NIDDM patients with vascular lesions and moderate to severe neuropathy (1st group). The similar NIDDM patients received "placebo" course (2nd group of breathing in ambient air in the same regimen). All the DM patients were glycemia compensated ($Hb A_{1c}=7.29\pm0.21\%$) with sugar controlling drugs perorally (sulfonylurea group drugs). A hypoxic gas mixture (HGM) produced by the "Hypoxicator" apparatus was used containing 11.5% O_2 for breathing through a mask with ambient air intervals. One session lasted for 30 minutes of HGM breathing, one course consisted of 20 sessions, one session a day. The IHT was well tolerated and no side effects were observed compared with placebo. The results are presented in the table.

№	Indices	NIDDM patients			
		The IHT group; n=40		The placebo group; n=20	
		Before IHT	After IHT	Before IHT	Before IHT
1	Glucose, mmol/l	6,44±0,16	6,39±0,21	6,58±0,18	6,59±0,21
2	Insulin resistance, $\mu U/ml$	24,35±1,14	14,2±1,12*	26,84±1,35	24,78±2,13
3	Erythropoietin, mU/ml	6,18±0,75	17,2±3,15*	7,12±0,92	8,11±1,13
4	Lactate, mmol/l	2,42±0,15	1,91±0,10*	2,62±0,24	2,51±0,18
5	Pyruvate, $\mu mol/l$	74,0±3,05	64,9±1,48*	72,8±4,12	71,3±1,55
6	CV Sensitivity (crus), U	2,2±0,04	1,2±0,20*	2,4±0,40	2,4±0,30
* p<0.05					

Conclusion: The IHT has a beneficial effect on the oxygen transport system, reduces insulin resistance, and neuropathy.

DOES HYPOXIA DURING RECOVERY FROM FATIGUING EXERCISE EFFECT PLASMA LACTATE DECAY CURVES?

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The aim of this study was to investigate the effect of breathing hypoxic gas during recovery on plasma lactate (Lac) disappearance in well-trained runners. 11 runners, 6 African (A) and 5 Caucasian (C), (age 23 ± 3.9 yr and best 10 km race time $32:10 \pm 1:20$ min:s) participated in this study. On separate days each athlete performed a VO_2max in normoxic conditions (Nor, 21% O_2) and normobaric hypoxia (Hyp, 14% O_2). On two more visits, each athlete did an incremental exercise test in Norm and Hyp, exercising for 5 min at workloads of 64%, 72% and 80% of peak treadmill speed (PTS) achieved in the appropriate maximal test, plus 88% PTS to fatigue. Blood samples were obtained during recovery for later determination of Lac. Mono-exponential curves were fitted to each subject's Lac data (mean R^2 for 22 curves was 0.92 ± 0.06). VO_2max in Norm and Hyp was 68.9 ± 5.76 and 45.2 ± 10.2 mmol/kg/min respectively. PTS was 21.2 ± 1.5 km/h in Norm and 17.6 ± 1.4 km/h in Hyp. Mean Lac (mmol/L) 3 min into recovery was 7.6 ± 3.1 in Norm, and 8.0 ± 3.1 in Hyp; whereas after 18 min it was 3.9 ± 2.1 in Norm and 4.5 ± 1.8 in Hyp. Lac decay rate was significantly slower in Hyp than Norm ($P < 0.05$). Dividing the subjects into two groups (A and C) showed that Hyp did not affect Lac decay rate in C (NS) but that it was significantly ($P < 0.001$) slower in A in Hyp than Norm. Larger subject numbers are required to reach a definitive conclusion, but it would appear that normobaric hypoxia of 14% O_2 delays plasma lactate disappearance rate during recovery from fatiguing exercise, and that this response to hypoxia is most evident in subjects of Southern African origin. This may point to a parsimonious metabolism in the face of reduced oxygen supply in this group.

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